

Protein molecular structures in alfalfa hay cut at three stages of maturity and in the afternoon and morning and relationship with nutrient availability in ruminants

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Abstract

BACKGROUND: Molecular structures in feed protein influence its digestive behavior, availability and utilization. From a nutritive point of view, stage of maturity and cutting time are important factors affecting nutrient profiles and availability of alfalfa (*Medicago sativa* L.) hay in ruminants. The objectives of this study were to determine protein molecular structures by Fourier transform infrared spectroscopy (FTIR), and their relationship with nutrient profiles and availability in ruminants of alfalfa hay cut at early bud, late bud and early flower stages and in afternoon and morning.

RESULTS: With advancing maturity, molecular structure ratios of α -helix: β -sheets decreased, while amide I:amide II increased ($P \leq 0.05$). Alfalfa cutting in afternoon versus morning increased protein structure α -helix: β -sheets and α -helix:others ratios ($P < 0.05$) and tended to decrease the proportion of β -sheets ($P = 0.09$). Positive correlations were found for α -helix: β -sheet ratio ($R \geq 0.60$; $P < 0.05$) with intermediately degradable protein (PB2) and ruminal degradability and intestinal protein supply, and all these parameters correlated negatively with amide I:amide II ratio ($R \leq -0.62$; $P < 0.05$).

CONCLUSION: Protein molecular structures in alfalfa hay changed with advancing maturity and during the day and these protein structures affected predicted nutrient availability of alfalfa hay in ruminants.

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Keywords: alfalfa hay; cutting time; FTIR spectroscopy; protein molecular structures; stage of maturity

INTRODUCTION

The feeding value of plant proteins is closely related not only to total protein and amino acid content, but also to protein molecular structures.¹ Protein secondary molecular structures consist mainly of α -helix, β -sheets and small amounts of β -turn and random coil.^{2–4} The percentage of these structures influences protein quality, availability and digestive behavior. A high percentage of β -sheets is usually related to reduced access for gastrointestinal digestive enzymes to the protein, resulting in lower nutritive value compared with protein with a high percentage of α -helix.¹ Traditional 'wet' chemical analysis gives accurate information about the nutritional composition but cannot reveal protein internal structures and its relationship to nutritive quality and availability. Fourier transform infrared (FTIR) microspectroscopy is a quick, direct and non-destructive analytical technique which can reveal molecular structural features of biological samples.^{1,5,6}

Cultivated alfalfa (*Medicago sativa* L.) is one of the major forage crops in the world.⁷ Leaf:stem ratio, crude protein (CP) fractions, digestive behavior and availability of alfalfa hay are mainly influenced by stage of maturity (SM)^{8–11} and cutting time (CT) due to accumulation of non-structural carbohydrates and true protein fraction during the day as a result of photosynthesis.^{10–13} To date,

none of the published studies in the literature have investigated the effect of SM and CT on protein molecular structures of alfalfa hay and its relationship to protein digestibility and availability. Therefore, the primary objectives of this study were to determine protein molecular structures (primary and secondary features) by FTIR spectroscopy, to quantify protein inherent structures using Gaussian and Lorentzian multi-component peak modeling methods, and to discriminate protein molecular structures using principal component analysis in alfalfa hay cut at three SM (early bud, late bud and early flower) and two CT (in afternoon and morning). Secondly, we correlated these protein molecular

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structures with basic chemical composition (related to protein), CP fractions, *in situ* ruminal degradability and metabolizable protein supply in ruminants.

MATERIAL AND METHODS

Alfalfa plot management

A second-year alfalfa field (20 × 24 m) seeded with cv. Ranger at the Research Farm of Ferdowsi University of Mashhad (Mashhad, Iran; 36°17'52.8" N, 59°36'20.52" E) was used in this study. The whole field was harvested before the experiment on 6 April 2010 and irrigated every 10 days during the experiment.

Six plots (each 4 × 4 m) within five replicate blocks in a field were randomly assigned to six treatments in a factorial arrangement (3 SM × 2 CT). Alfalfa was cut at early bud (15/16 June), late bud (26/27 June) and early flower (18/19 July) both in the afternoon (18:00 h) and the following morning (06:00 h). The SM was determined according to Kalu and Fick.¹⁴ Briefly, a quadrat (250 cm²) was randomly thrown into each plot (one time) and all stems above 3 cm stubble height inside the quadrat (~70–80 stems) were used to calculate the mean SM for each plot. In total, there were 10 plots for each SM, from which half was cut at 18:00 h and the other half at 06:00 h, when alfalfa reached the appropriate SM as described in more detail by Yari *et al.*^{10,11} At each harvest, an area of 3 × 3 m was manually clipped using a small scythe at ~5 cm above the soil surface.

Alfalfa harvested from each plot was air dried in the shade for 10–15 days. After air drying, alfalfa hay samples were chopped using a hay chopper with a 20 mm screen (Agri-Equip, Nasr Co., Isfahan, Iran). This is the common procedure for processing alfalfa hay before mixing it in total mixed ration for dairy cows. The hay from the first, second and third blocks were pooled to one sample and hay from the fourth and fifth blocks were pooled to another sample to generate sufficient material for chemical analysis, *in situ* degradability and FTIR spectroscopy measurements.

Molecular structures analyzed by FTIR vibration spectroscopy

This part of the study was performed in the Department of Animal and Poultry Science, University of Saskatchewan (Saskatoon, SK, Canada). The alfalfa hay samples were finely ground twice to pass through a 0.2 mm screen (Retsch ZM-1, Brinkmann Instruments Ltd, Ontario, Canada). FTIR vibration spectroscopy was performed using a JASCO FT/IR-4200 with a ceramic infrared (IR) light source and a deuterated L-alanine doped triglycine sulfate detector (JASCO Corp., Tokyo, Japan) equipped with a MIRacle™ attenuated total reflectance accessory module and fitted with a ZnSe crystal and pressure clamp (PIKE Technologies, Madison, WI, USA). Spectra were generated from the mid-IR region (4000–800 cm⁻¹) using JASCO Spectra manager II software with a spectral resolution of 4 cm⁻¹ (Fig. 1A). Functional spectral bands associated with protein molecular structures were assigned according to published studies^{1,5,6} and identified with OMNIC 7.2 software (Spectra Tech, Madison, WI, USA) after noise elimination by JASCO Spectra manager II software.

Unique primary protein features found in peptide bonds (C—O, C—N and N—H) include amide I (~80% C=O and ~20% C—N stretching vibration; centered at a wavelength of ~1655 cm⁻¹) and amide II (~60% N—H bending vibration, ~40% C—N stretching vibration; centered at ~1550 cm⁻¹), within the wavelength region from ~1720 to 1485 cm⁻¹ (Fig. 1A,B).^{2–6}

The amide I peak consists of multi-components of the protein secondary structure which overlap. These multi-components

include α -helix, β -sheets, random-coil and β -turn (Fig. 1C).¹ Therefore, a specific multi-peak-fitting/modeling procedure was applied to reveal protein secondary structures within amide I component bands in alfalfa hay (Figs 1C and 2A,B). Two steps were performed to determine the relative amount of α -helix, β -sheets and other protein internal structures (e.g. random coils, β -turns) within this amide I band. The first step was to use the Fourier self-deconvolution method (FSD) to resolve overlapping bands^{15,16} using OMNIC (Fig. 1C; Spectra Tech) in the spectra range from 1707 to 1563 cm⁻¹. This identifies protein component peak frequencies in the amide I region (Fig. 1C). In the second step, model relative multi-component peak areas were calculated from FSD spectra by the Gaussian and Lorentzian functions using the multi-peak-fitting program in Origin (v6.1052, Origin Lab Corp., Northampton, MA, USA) to generate usable model curves as in Yu¹⁷ (Fig. 2). The Gaussian and Lorentzian functions (left and right equations, respectively) are expressed as follows:

$$y = y_0 + \frac{A}{w\sqrt{\pi}/2} e^{-\frac{2(x-x_c)^2}{w^2}} \quad \text{and} \quad y = y_0 + \frac{2A}{\pi} \frac{w}{4(x-x_c)^2 + w^2}$$

where y_0 , x_c , w and A represent peak offset, peak center (at the peak maximum), peak width and peak area, respectively (Fig. 2). The peaks (shape, center, offset, width and areas) and the relative proportion of α -helix, β -sheets and other structural features based on modeled peak areas were calculated by both the Gaussian and Lorentzian function in origin. In the current study, the relative modeled peak area for α -helix and β -sheets were found at ~1656 and 1633 cm⁻¹ respectively.

Alfalfa protein was also fractionated by traditional wet chemistry and *in situ* Nylon bag incubations in combination with modeling protein availability along the gastrointestinal tract which were described in detail by Yari *et al.*^{10,11} In short, firstly alfalfa protein was chemically fractionated into five fractions based on ruminal degradation characteristics according to the Cornell Net Carbohydrate and Protein System (CNCPS);^{10,18} secondly, alfalfa was incubated in Nylon bags in the rumen of three cows for up to 72 h and protein fractionated into a washout, potentially degradable and undegradable fraction and protein residues over time fitted to an exponential model;¹¹ thirdly, chemical and Nylon bag output data were used to calculate protein degradation characteristics and nutrient supply in dairy cows with the DVE/OEB protein evaluation system.¹⁹

Statistical analysis

Spectral data collected by FTIR is usually analyzed by univariate and multivariate methods.¹ The univariate analysis of data from chopped alfalfa hay samples from two pooled blocks used to test the effect of SM and CT on protein primary and secondary structures was performed using the PROC MIXED procedure of SAS 9.2²⁰ with the following statistical model:

$$Y_{ijk} = \mu + B_k + CT_i + SM_j + CT_i \times SM_j + e_{ijk}$$

where Y_{ijk} is the observation of the dependent variables ijk ; μ is the fixed effect of population mean for the variable; B_k is the random effect of pooled block ($k = 2$); CT_i is the fixed effect of cutting time ($i = 2$; 06:00 h and 18:00 h); SM_j is the fixed effect of stage of maturity ($j = 3$; early bud, late bud and early flower); $CT_i \times SM_j$ is the fixed effect of interaction between factor CT at level i and the factor SM at level j ; and e_{ijk} is the random error associated with the observation ijk . Multi-treatment comparisons were performed

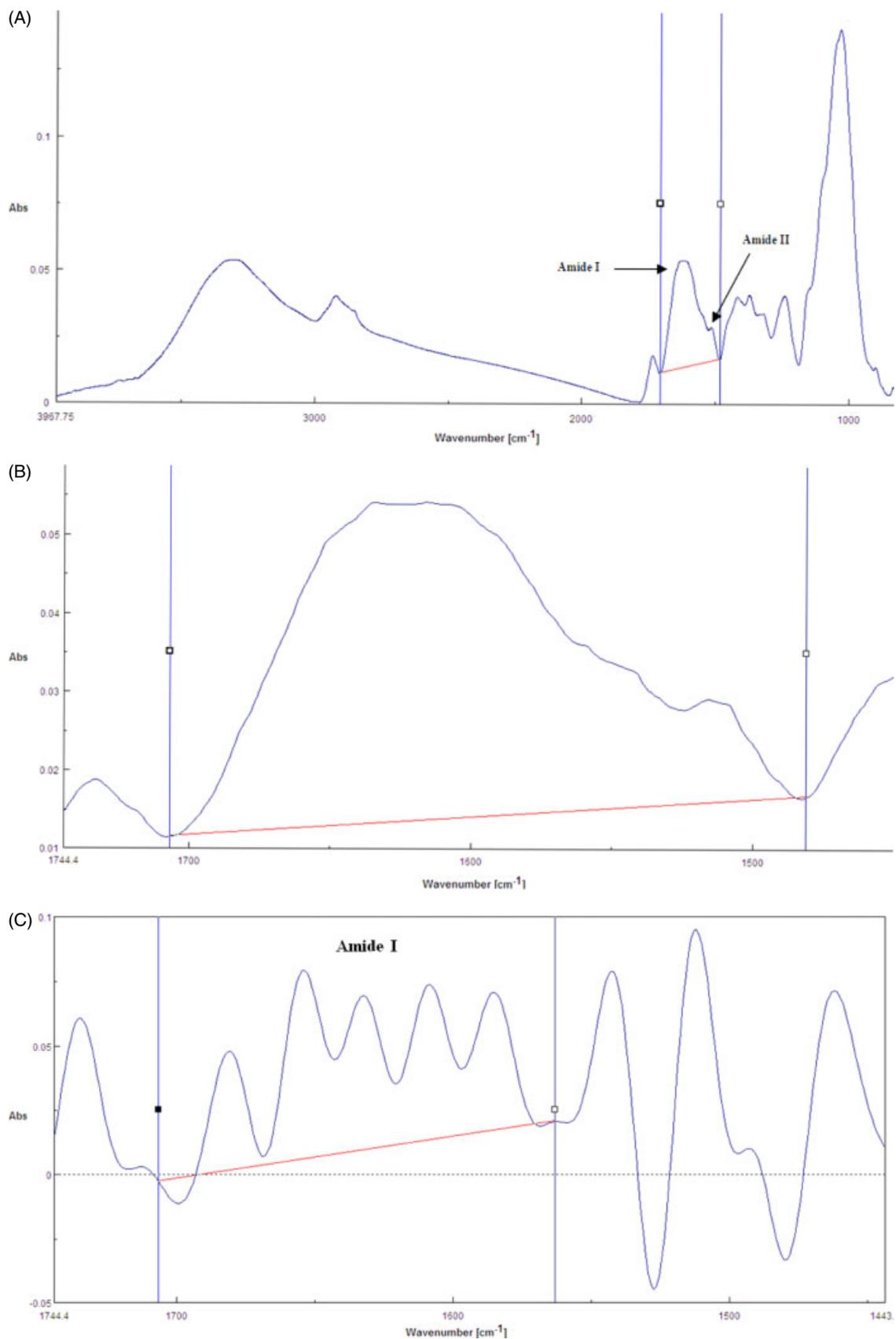


Figure 1. Typical full-range spectrum, amide I and II areas and Fourier self-deconvolution of alfalfa hay: (A) peak area of amide I ($\sim 1707\text{--}1563\text{ cm}^{-1}$) and amide II ($\sim 1563\text{--}1481\text{ cm}^{-1}$); (B) enlargement of amide I and II area; (C) Fourier self-deconvolution spectrum of amide I area revealing overlapped peak frequencies.

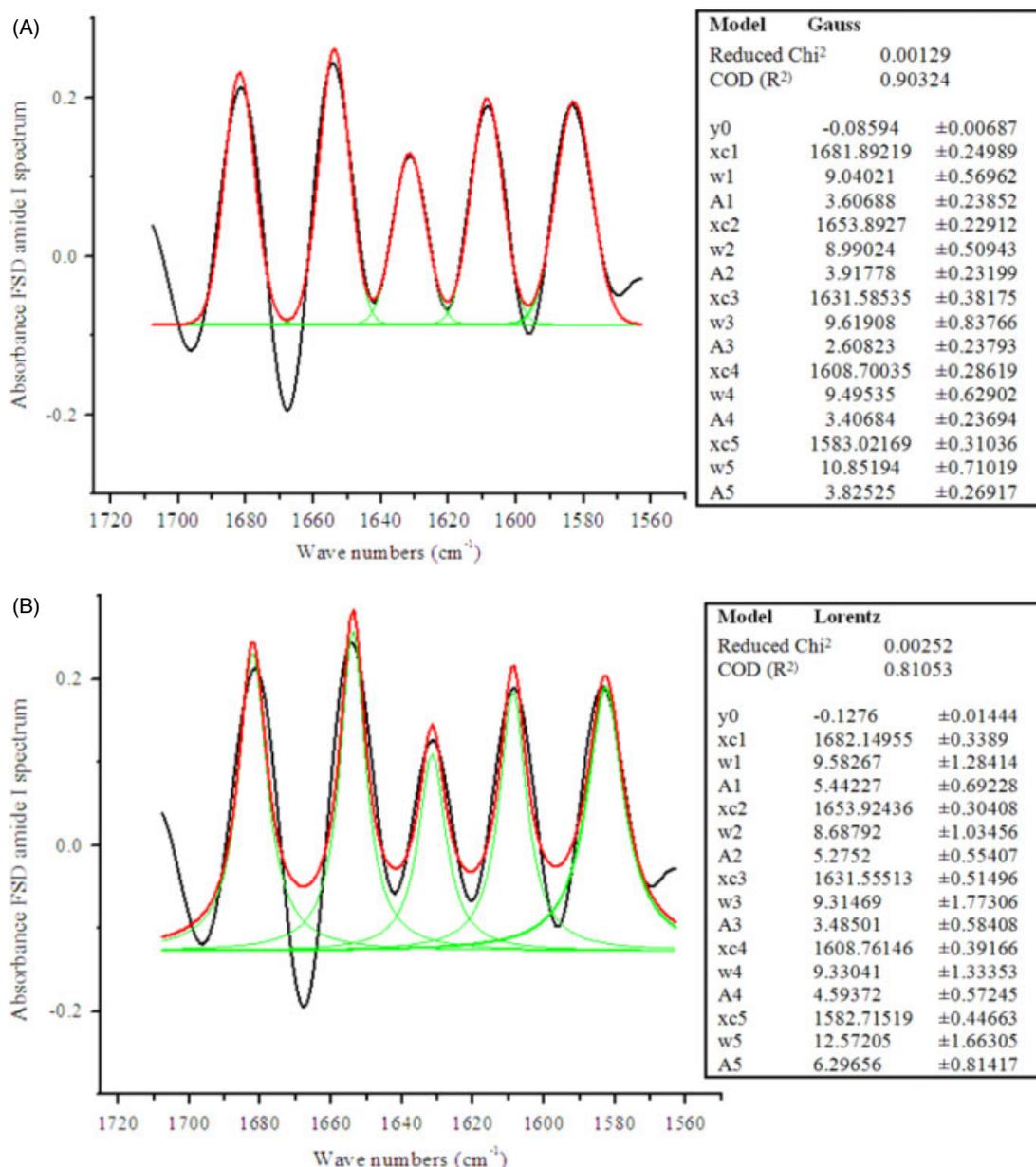


Figure 2. Multi-component peak modeling in origin of a typical Fourier self-deconvolution (FSD) spectrum in the protein amide I region (~1707–1563 cm⁻¹) of alfalfa hay to find proportion of α -helix and β -sheets: (A) Gaussian modeling method showing 23% model-fitted α -helix and 15% model-fitted β -sheets; (B) Lorentzian modeling method showing 21% model-fitted α -helix and 14% model-fitted β -sheets.

with Fisher's protected least significant difference (LSD) test by the LSMEAN statement of SAS 9.2²⁰ with letter groupings obtained using the SAS pdmix800 macro. For the different statistical tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$, unless otherwise stated.

Simple statistics (mean, maximum, minimum and standard deviation) for the nutrient profiles and availability were given (detailed results published in Yari *et al.*^{10,11}). Correlation between protein molecular structures with nutrient profiles and availability in ruminants for alfalfa hays tested ($n = 12$) were analyzed using the CORR procedure of SAS 9.2²⁰ with Pearson's correlation coefficient (R).

Multivariate data analysis was performed on the whole spectra of the amide region (~1707–1483 cm⁻¹) using cluster analysis and principal component analysis in Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) as described in detail by Yu.²¹

RESULTS

Effect of alfalfa stage of maturity and cutting time on protein molecular structures

Alfalfa at early bud had higher amide I compared with alfalfa at early flower stage ($P < 0.05$), with alfalfa at late bud intermediate (Table 1). Alfalfa at early and late bud had higher amide II and lower amide I:amide II ratio compared with alfalfa at early flower stage ($P < 0.05$; Table 1).

The Gaussian multi-peak modeling method fitted better to peak areas from FSD spectra of alfalfa hay tissue than the Lorentzian method, as expressed by a higher fitting R^2 value (0.90 vs. 0.81, respectively; Fig. 2A,B). Based on the Gaussian method, alfalfa hay at early bud had lower β -sheets and β -sheets:others ratio compared with alfalfa hay at early flower stage, with alfalfa at late bud intermediate ($P < 0.05$; Table 2). Alfalfa hay at early bud had

Table 1. Brief description and FTIR spectroscopic vibration intensities associated with protein primary molecular structures of alfalfa hay cut at three stages of maturity and in the afternoon and morning

Items	Stage of maturity (SM)			SEM	Cutting time (CT)		SEM	P-value ^a	
	Early bud	Late bud	Early flower		18:00	06:00		SM	CT
Summary of alfalfa hays ^b									
Yield (g m ⁻²)	337c	470b	537a	14.0	449	447	11.4	<0.01	0.90
Crude protein (g kg ⁻¹ DM)	220a	195b	162c	4.8	192	192	3.9	<0.01	0.96
RDP (g kg ⁻¹ DM)	135a	116b	86c	4.2	112	113	3.4	<0.01	0.89
RUP (g kg ⁻¹ DM)	85a	79b	76b	2.9	80	80	2.8	<0.01	0.81
Protein primary molecular structures of alfalfa hays ^c									
Amide I	3.55a	3.33ab	3.02b	0.110	3.28	3.32	0.09	0.03	0.78
Amide II	1.24a	1.18a	0.99b	0.061	1.13	1.14	0.05	0.03	0.97
Amide I:amide II	2.86b	2.84b	3.07a	0.050	2.91	2.93	0.04	0.02	0.74

^a There was no interaction between stage of maturity and cutting time; SEM, standard error of mean; means with different letters (a, b and c for SM and d and e for CT) within the same row differ ($P < 0.05$).

^b RDP, ruminal-degradable protein; RUP, ruminal-undegradable protein calculated based on NRC (2001) as described in Yari *et al.*¹¹

^c Vibration intensity absorbed due to protein structures (baseline 1707–1483 cm⁻¹) with the peak region for amide I at 1707–1563 cm⁻¹ and for amide II at 1563–1483 cm⁻¹.

Table 2. Effect of stage of maturity (EB, early bud; LB, late bud and EF, early flower) and cutting time (18:00 and 06:00 h) on the Gaussian model-fitted protein structure profiles of alfalfa hay ($n = 60$)

	Stage of maturity (SM)			SEM	Cutting time (CT)		SEM	P-value ^a	
	EB	LB	EF		18:00	06:00		SM	CT
Model-fitted protein structure profile									
α -helix	0.2086a	0.1998b	0.1998b	0.00225	0.2070d	0.1978e	0.00188	0.05	0.02
β -sheets	0.1623c	0.1745b	0.1866a	0.00198	0.1721	0.1769	0.00161	<0.01	0.09
Others	0.6290a	0.6257a	0.6146b	0.00257	0.6209e	0.6253d	0.00144	<0.01	0.02
Protein structure ratio profile									
α -helix: β -sheets	1.2873a	1.11475b	1.0655b	0.02429	1.2113e	1.1223d	0.01983	<0.01	0.02
α -helix:others	0.3319	0.3194	0.3234	0.00419	0.3333d	0.3164e	0.00359	0.15	0.01
β -sheets:others	0.2580c	0.2789b	0.3036a	0.00330	0.2773	0.2830	0.00269	<0.01	0.20

^a There was no interaction between stage of maturity and cutting time; SEM, standard error of mean; Means with different letters (a, b and c for the SM and d and e for the CT) within the same row differ ($P < 0.05$).

higher α -helix and α -helix: β -sheets ratio compared with alfalfa hay at late bud and early flower stage ($P < 0.05$; Table 2). Alfalfa hay at early and late bud had higher other protein molecular structures compared with alfalfa hay at early flower ($P < 0.05$; Table 2). Alfalfa hay cutting in the afternoon *versus* morning increased α -helix, α -helix: β -sheets ratio and α -helix:others ratio ($P < 0.05$) and decreased other protein structural features ($P < 0.05$) and tended to decrease β -sheets ($P = 0.09$; Table 2).

Correlation of protein molecular structures with protein fractions, ruminal protein degradation and predicted protein supply

The α -helix: β -sheets and α -helix:others ratios correlated positively ($P < 0.05$; $R \geq 0.60$) with total CP, intermediate degradable protein (PB2), buffer-soluble CP (BSCP), *in situ* potentially degradable CP (D_{CP}) and rumen-degradable protein (RDP), while all of these components correlated negatively ($P < 0.10$; $R = -0.51$ to -0.85) with amide I:amide II and β -sheets:others ratio (Table 3). The α -helix: β -sheets ratio correlated negatively ($P = 0.05$; $R = -0.58$) and amide I:amide II and β -sheets:others ratio positively ($P < 0.01$; $R \geq 0.66$) with *in situ* undegradable CP and rumen-undegradable protein (RUP; Table 3). Additionally, amide I:amide II ratio correlated positively ($P = 0.05$; $R = 0.57$) with neutral detergent-insoluble CP (NDICP) and α -helix: β -sheets ($P = 0.06$; $R = -0.55$)

and α -helix:others ratio ($P = 0.02$; $R = -0.64$) negatively with acid detergent-insoluble CP (ADICP) (Table 3).

The α -helix: β -sheets and α -helix:others ratios had a positive correlation with the model-predicted parameters absorbed microbial crude protein (AMCP), absorbed RUP (ARUP), truly intestinally absorbed protein (DVE), fermentable organic matter (FOM), degraded protein balance (OEB), MCP from FOM (MCP_{FOM}), MCP_{RDP} and total supplied intestinally protein (TSPI) ($P < 0.10$; $R \geq 0.52$), whereas all of these components were negatively correlated with amide I:amide II and β -sheets:others ratios ($P < 0.10$; $R < -0.56$; Table 4). The α -helix: β -sheets ($P = 0.02$ and $R = -0.66$) and α -helix:others ratios ($P = 0.06$ and $R = -0.56$) had a negative correlation with predicted endogenous protein losses (ENDP) and undegradable dry matter (UDM), whereas these components correlated positively with amide I:amide II ($R = 0.79$) and β -sheets:others ratios ($R = 0.73$; $P < 0.01$; Table 4).

DISCUSSION

Effect of stage of maturity and cutting time

This study identified the effect of SM on FTIR vibration intensities related to protein molecular structures in alfalfa hay. We used cluster and principal component analysis to discriminate (or not) in the IR spectrum in the amide region (~ 1707 – 1483 cm⁻¹) among

Table 3. Summary of crude protein fractions and *in situ* crude protein degradation kinetics of alfalfa hay (cut at three stages of maturity and two cutting times; total sample number = 12) and their correlations with primary and secondary protein molecular structures

items	Mean	Range			Amide I:amide II		α -helix: β -sheets		α -helix:others		β -sheets:others	
		Minimum	Maximum	SD ^a	R	P-value	R	P-value	R	P-value	R	P-value
CP (g kg ⁻¹ DM)	192	154	225	26.2	-0.84	<0.01	0.80	<0.01	0.66	0.02	-0.88	<0.01
Chemical composition related to crude protein (g kg ⁻¹ CP) ^b												
NDICP	129	108	162	15.8	0.57	0.05	-0.22	0.48	-0.27	0.40	0.19	0.54
ADICP	69	50	93	14.1	0.32	0.30	-0.55	0.06	-0.64	0.02	0.42	0.17
BSCP	421	391	474	23.1	-0.51	0.09	0.68	0.02	0.74	<0.01	-0.52	0.09
TP	542	491	633	37.3	-0.14	0.64	0.23	0.45	0.36	0.25	-0.08	0.80
Protein fractions (g kg ⁻¹ CP) according to Cornell net carbohydrate and protein system (CNCPS) ^c												
PA (i.e. NPN)	458	367	509	37.3	0.15	0.65	-0.23	0.43	-0.36	0.25	0.08	0.80
PB	473	406	561	43.8	-0.22	0.47	0.38	0.22	0.51	0.09	-0.21	0.52
PB1	121	98.3	200	28.5	0.22	0.49	-0.24	0.45	-0.13	0.68	0.31	0.30
PB2	291	250	343	28.4	-0.74	<0.01	0.68	0.02	0.75	<0.01	-0.53	0.08
PB3	60	17	80	19.1	0.23	0.46	0.22	0.49	0.25	0.42	-0.16	0.64
PC	69	50	93	14.1	0.32	0.30	-0.55	0.06	-0.64	0.02	0.42	0.17
<i>In situ</i> degradation kinetics of crude protein (g kg ⁻¹ CP) ^d												
W _{CP}	189	110	289	52.2	-0.21	0.50	-0.12	0.69	-0.12	0.69	0.09	0.77
D _{CP}	533	420	618	55.0	-0.54	0.07	0.63	0.02	0.53	0.07	-0.66	0.02
U _{CP}	278	207	370	47.9	0.84	<0.01	-0.58	0.05	-0.47	0.12	0.66	0.02
Kd _{CP} (h ⁻¹)	0.13	0.07	0.16	0.03	-0.18	0.57	0.47	0.12	0.51	0.09	-0.36	0.24
RDP	554	452	633	50.1	-0.62	0.03	0.66	0.02	0.54	0.07	-0.71	<0.01
RUP	421	366	491	41.9	0.85	<0.01	-0.69	0.01	-0.62	0.03	0.70	0.01

^a SD, standard deviation; R, correlation coefficient.

^b CP, crude protein; ADICP, acid detergent-insoluble CP; NDICP, neutral detergent-insoluble CP; BSCP, buffer-soluble CP; TP, true protein (CP-NPN).

^c Protein fractions according to CNCPS¹⁸ including PA, instantaneously solubilizable CP (i.e. NPN); PB1, soluble true protein (BSCP-NPN); PB2, intermediate degradable true protein [CP - (PA + PB1 + PB3 + PC)]; PB3, slowly degradable true protein (NDICP - ADICP); PC, undegradable protein (i.e. ADICP).

^d *In situ* CP fractions: W, washout fraction; D, potentially degradable fraction; U, undegradable fraction; Kd, fractional degradation rate; RDP, rumen-degradable protein; RUP, rumen-undegradable protein.

Table 4. Summary of predicted nutrient supply to ruminants of alfalfa hay (cut at three stages of maturity and two cutting times; total sample number = 12) and their correlation with protein molecular structures

Item	Mean	Range			Amide I:amide II		α -helix: β -sheets		α -helix:others		β -sheets:others	
		Minimum	Maximum	SD ^a	R	P-value	R	P-value	R	P-value	R	P-value
Predicted nutrient supply to dairy cows using DVE/OEB model (g kg ⁻¹ DM) ^b												
AMCP	44	38	49	3.0	-0.65	0.02	0.52	0.08	0.45	0.14	-0.56	0.06
ARUP	32	21	46	6.9	-0.74	<0.01	0.71	0.01	0.54	0.07	-0.81	<0.01
DVE	49	26	68	11.3	-0.78	<0.01	0.71	<0.01	0.57	0.05	-0.80	<0.01
ENDP	27	3	23	32.9	0.79	<0.01	-0.66	0.02	-0.56	0.06	0.73	<0.01
FOM	462	402	508	26.8	-0.65	0.02	0.52	0.08	0.45	0.14	-0.56	0.06
OEB	34	7	57	20.1	-0.80	<0.01	0.77	<0.01	0.65	0.02	-0.82	<0.01
MCP _{FOM}	69	60	76	4.2	-0.65	0.02	0.52	0.08	0.45	0.14	-0.56	0.06
TSPI	141	129	152	6.9	-0.62	0.03	0.73	<0.01	0.56	0.06	-0.85	<0.01
MCP _{RDP}	103	70	132	22.0	-0.83	<0.01	0.78	<0.01	0.67	0.02	-0.84	<0.01
UDM	362	307	441	35.1	0.79	<0.01	-0.66	0.02	-0.56	0.06	0.73	<0.01

^a SD, standard deviation; R, correlation coefficient.

^b Nutrient supply parameters were calculated according the DVE/OEB 1994 protein evaluation system^{11,19} with the following parameters: AMCP, intestinal absorbed MCP; ARUP, intestinal absorbable rumen undegradable protein; DVE, truly digested and absorbed protein in the intestine; ENDP, endogenous protein losses in the digestive tract; FOM, fermentable organic matter; MCP_{FOM}, microbial crude protein synthesized in the rumen from FOM; MCP_{RDP}, MCP synthesized in the rumen from RDP; OEB, rumen-degraded protein balance; TSPI, true protein supplied to the small intestine; UDM, completely undegradable DM.

With advancing maturity, α -helix: β -sheets decreased, β -sheets:others increased and α -helix:others remained similar. Alfalfa hay in the current study had lower average α -helix: β -sheets, α -helix:others and β -sheets:others ratios of 1.17, 0.30 and 0.26, respectively, compared with 1.35, 0.80 and 0.63, respectively, reported for alfalfa leaves at a vegetative stage.²² These results suggest that alfalfa in our study contained less α -helix relative to β -sheets and others and less β -sheets relative to others than alfalfa in Yu *et al.*²² Differences in protein secondary structures between our study and Yu *et al.*²² might have resulted from the different sample fractions used (whole forage vs. leaves), different sample drying method used (air dried in the shade vs. freeze drying), stage of maturity at harvest (three stages of maturity vs. vegetative) and we scanned whole ground plant material, while Yu *et al.*²² scanned intact leaf cross-sections using synchrotron FTIR, which allows focusing on protein-rich areas (less interference with other compounds in the feed). Leaf:stem ratio of fresh alfalfa, which was used in its dried form in this study, reduced with advancing maturity,¹⁰ as was the case in other studies.^{23–26} The α -helix: β -sheets ratio also increased with advancing maturity, and β -sheets:others ratios decreased. These results suggest that protein in alfalfa leaves contains more α -helix relative to β -sheets and others and less β -sheets relative to others than protein in stems. This could be the main reason for the differences in protein molecular structures in our study compared with Yu *et al.*²² Leaf protein consists mainly of photosynthetic enzymes (RUBISCO) and stem proteins consist mainly of fiber-related proteins.^{10,24} These different main proteins in leaves and stems probably cause the difference in secondary structures between them.

Alfalfa cut in the afternoon had higher α -helix: β -sheets and α -helix:others ratios compared with alfalfa cut in the morning, while other protein molecular structure ratios were similar between both CT. This trend is similar to previous findings for alfalfa at vegetative stage cut in the evening and morning.²⁴ In a previous study¹⁰ with current samples we found that cutting alfalfa in the afternoon *versus* morning increased intermediately degradable protein (PB2; CNCPS basis) and decreased instantaneously solubilized CP fraction (PA; CNCPS basis). But cutting time did not affect *in situ* protein degradation characteristics or protein availability to the animal.¹¹

Correlation between protein molecular structures and nutrient profiles and availability

The RDP, MCP, OEB, RUP, and DVE decreased with advancing maturity,¹¹ while these components were similar between alfalfa hay cut in the afternoon and morning. Amide I:amide II and β -sheets:others ratios correlated negatively and α -helix: β -sheets and α -helix:others ratios correlated positively with RDP, FOM, MCP based on FOM and RDP, OEB, AMCP, ARUP and DVE in this study, while correlations of these protein molecular structures were opposite with NDICP and *in situ* U_{CP} . Furthermore α -helix: β -sheets and α -helix:others ratios correlated negatively with ADICP. Thus amide I:amide II and β -sheets:others ratios correlated negatively and α -helix: β -sheets and α -helix:others ratios correlated positively with protein metabolism in both the rumen and the intestine of dairy cows.

Previously, an alfalfa cultivar selected for low initial rate of ruminal degradation was found to have higher α -helix: β -sheets ratio, U_{CP} and U_{OM} and lower amide I:amide II ratio, RDP and DVE compared with anthocyanidin-accumulating alfalfa.^{24,27} Hullless barley cultivars selected for different starch characteristics had negative correlation between α -helix: β -sheets ratio and DVE and

positive correlation between amide I:amide II ratio and RDP and OEB.²⁸ The α -helix: β -sheets ratio correlated negatively with RDP and OEB and positively with ARUP and DVE in flax seed exposed to different heat treatments (correlations for amide I:amide II ratio not shown).²⁹ Amide I:amide II ratio correlated positively with OEB and negatively with AMCP, intestinal RUP digestibility and U_{CP} in different bio-ethanol co-products, while none of these parameters correlated with α -helix: β -sheets ratio.³⁰ Liu³¹ found that both amide I:amide II and α -helix: β -sheets ratio correlated negatively with CP, RDP, OEB and intestinal RUP digestibility, and amide I:amide II ratio also correlated negatively with RUP, ARUP and DVE and positively with AMCP in different cereal grains and bio-ethanol co-products. In three of these studies,^{28–30} protein molecular structures correlated with predicted ruminal metabolism in the opposite direction rather than with predicted intestinal metabolism, while in the study of Liu,³¹ as well as in our study, protein molecular structures correlated in a similar direction for predicted protein metabolism in both the rumen and the intestine. The protein content in the original samples was similar among treatments in those three studies,^{28–30} while in our study and that of Liu³¹ there was a large range in CP content among the samples tested. This suggests that in our study and that of Liu³¹ overall CP content had a greater impact on predicted protein metabolism in the rumen and intestine than molecular structural makeup of the proteins in the feed.

CONCLUSION

The FTIR spectroscopy vibration intensities due to alfalfa hay protein molecular structures changed with maturity from early bud to early flower stage and between alfalfa hay cut in the afternoon and morning. Alfalfa hay protein molecular structures such as ratios of amide I:amide II, α -helix: β -sheets, α -helix:others and β -sheet:others influenced predicted protein quality, nutrient availability and digestive behavior in ruminant prediction models, but to a lesser extent than protein content.

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