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Using non-invasive molecular spectroscopic techniques to detect unique aspects of protein Amide functional groups and chemical properties of modeled forage from different sourced-origins

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ABSTRACT

The non-invasive molecular spectroscopic technique-FT/IR is capable to detect the molecular structure spectral features that are associated with biological, nutritional and biodegradation functions. However, to date, few researches have been conducted to use these non-invasive molecular spectroscopic techniques to study forage internal protein structures associated with biodegradation and biological functions. The objectives of this study were to detect unique aspects and association of protein Amide functional groups in terms of protein Amide I and II spectral profiles and chemical properties in the alfalfa forage (*Medicago sativa* L.) from different sourced-origins. In this study, alfalfa hay with two different origins was used as modeled forage for molecular structure and chemical property study. In each forage origin, five to seven sources were analyzed. The molecular spectral profiles were determined using FT/IR non-invasive molecular spectroscopy. The parameters of protein spectral profiles included functional groups of Amide I, Amide II and Amide I to II ratio. The results show that the modeled forage Amide I and Amide II were centered at 1653 cm^{-1} and 1545 cm^{-1}, respectively. The Amide I spectral height and area intensities were from 0.02 to 0.03 and 2.67 to 3.36 AI, respectively. The Amide II spectral height and area intensities were from 0.01 to 0.02 and 0.71 to 0.93 AI, respectively. The Amide I to II spectral peak height and area ratios were from 1.86 to 1.88 and 3.68 to 3.79, respectively. Our results show that the non-invasive molecular spectroscopic techniques are capable to detect forage internal protein structure features which are associated with forage chemical properties.

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1. Introduction

Alfalfa (*Medicago sativa* L.) is considered as an excellent high-protein forage in ruminant livestock systems (eg. lactating cows), having many unique characteristics, such as high productivity, good longevity, high nutrient level and high digestibility. Nowadays, dairy producers and industries pursue high quality milk by using alfalfa forage as their major forage source in dairy cow rations [1].

Biodegradation, biological function, and nutrient utilization of forage protein depend on internal protein molecular structure profile [1–5]. The conventional "wet" chemical analyses usually destroy the internal structure of forage feeds during the pre-treatment and digestion for total compound analysis. This makes it impossible to link the internal structure to forage biological functions.

The molecular spectroscopic technique-FT/IR is non-invasive and non-destructive technique. This technique is capable to detect the molecular structure spectral features that are associated with biological, nutritional and biodegradation functions [1–5].

Several studies have demonstrated that the molecular spectroscopy (FT/IR) technique is able to reveal the differences of internal structure among different seed resources [6,7]. To date, few researches have been conducted to systematically quantify the features of the molecular structures in the forage materials. Few researches have been conducted to use this non-invasive molecular spectroscopic techniques to study forage internal molecular structure associated with biodegradation and biological functions.

The objectives of this study were to detect unique aspects and association of protein inherent structure (in terms of the functional groups of Amide I and Amide II spectral profiles) and chemical properties in the alfalfa forage from different sourced-origins.

2. Materials and methods

2.1. Modeled forage for molecular structure study in terms of Amide functional groups

In this study, common alfalfa hay was used as a modeled forage for molecular spectroscopic study. Five different sources of the modeled forage from China (code as "CSO" n = 5) and seven different sources

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from foreign countries (code as "FOS" n = 7) transported to China were used in this study. The standard commercial management practices in terms of growth conditions and cutting stage/harvesting were applied to produce the alfalfa forage hay. Total 12 sources of alfalfa hay were obtained (5 sources of CSO + 7 sources of FOS).

2.2. Chemical property studies on modeled forage

All samples were ground through a 1 mm screen using a mill (KRT-34, Kunchen Yucheng Machine Equipment Co., Ltd.). The dry matter (AOAC official method 930.15), ash (AOAC official method 942.05), ether extract (AOAC official method 954.02), and crude protein (AOAC official method 984.13) were analyzed respectively following the procedures of the AOAC [8]. The non-protein nitrogen value was obtained by precipitating true protein in the filtrate with 10% sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and calculated as the difference between total crude protein and residual crude protein after filtration [9]. The total soluble crude protein content was obtained by incubating the sample with borate–phosphate buffer and filtering through filter paper according to the description by Roe et al. [10] The values of acid detergent fiber, neutral detergent fiber and acid detergent lignin were determined as the method described by Van Soest et al. [11] Sodium sulfite (Na_2SO_3) and heat-stable amylase were added during the analysis of neutral detergent fiber. The contents of neutral detergent-insoluble crude protein and acid detergent-insoluble crude protein were obtained by Kjeldahl-N analysis of the neutral detergent fiber (without the addition of sodium sulfite) and acid detergent fiber residues according to the method of Licitra et al. [9]. The starch was analyzed using the Megazyme Total Starch Assay Kit with 0.5 mm particle size (AOAC, official method 996.11). The anthrone-sulfuric acid colorimetric method was used to determine the soluble sugars [12] in this study. The total carbohydrate, non-fiber carbohydrate, hemicellulose, and cellulose were calculated according to the NRC dairy model [13].

2.3. Non-invasive spectroscopic technique

All modeled forage samples were ground using APS standard method for spectral analysis [1–6]. The molecular spectrum data were collected using Lambda FTIR-7600 with ATR (Tianjin Gangdong Sci. & Tech. Development Co. Ltd). The molecular structural features were collected in the middle infrared radiation region from ca. 4000–800 cm^{-1} with 64 co-added scans, 4 cm^{-1} spatial resolution and 8 replications for each modeled forage sample. The background spectrum was also collected to minimize the influence of ambient. For detailed procedure, please check references [1–5].

2.4. Univariate spectral analysis

For forage protein univariate molecular spectral analysis, the spectra were analyzed by OMNIC 7.3 software (Spectra Tech., Madison, WI, USA). The protein chemical functional spectral bands include the Amide I and Amide II [4,6,14]. The detected parameters in relation to the protein are as follow: (1) Amide I and Amide II peak height; (2) Amide I and Amide II peak area; (3) the height ratio of Amide I and Amide II; and (4) the area ratio of Amide I and II. The spectra were processed by both secondary derivative function and Fourier self-deconvolution (FSD) in OMNIC 7.3 software to obtain the FSD and 2nd derivative spectra which reveal the special location of the measured parameters.

2.5. Statistical analysis

2.5.1. Studies on chemical properties

The differences between the two sourced-origins of the modeled forage in chemical and nutrient profile were analyzed using the TTEST procedure of Statistical Analysis Systems 9.4 (SAS Institute inc., Cary, NC). The model was: $Y_{ij} = \mu + F_i + e_{ij}$, where, Y_{ij} was an observation of the dependent variable ij; μ was the population mean for the variable; F_i was the fixed effect of the sourced-origins of the modeled forage (i = 1, 2), the sources of modeled forage in each sourced-origin as replications (five different sources of the modeled forage from China, code as "CSO" n = 5 and seven different sources from the foreign countries, code as "FOS" n = 7), and e_{ij} was the random error associated with the observation ij. Difference significance was declared if $P \le 0.05$, while trends were discussed at $0.05 < P < 0.10$.

2.5.2. Protein Amide functional groups study

The statistical analysis of forage protein functional group spectral data was carried out using a completely randomized design with sub-sampling model with Proc Mixed of Statistical Analysis Systems 9.4 (SAS Institute inc., Cary, NC) for mean, standard error of mean (SEM) and P-value. The model was: $Y_{ij} = \mu + F_i + S(F)_j + e_{ij}$, where, Y_{ij} was an observation of the dependent variable ij; μ was the population mean for the variable; F_i was the fixed effect of the sourced-origins of the modeled forage (i = 1, 2), five to seven sources of modeled forage in each sourced-origin as replications (five different sources of the modeled forage from China, code as "CSO" n = 5 and seven different sources from the foreign countries, code as "FOS" n = 7); $S(F)_j$ was an effect of the sources of modeled forage nested with the each sourced-origin, and e_{ij} was the random error associated with the observation i. Difference significance was declared if $P < 0.05$, while trends were declared at $0.05 < P < 0.10$.

3. Result and discussion

3.1. Detect unique aspects and association of forage chemical properties

Results of the magnitude of difference in chemical profiles of the modeled forage between two different sourced-origins (CSO vs. FSO) are shown in Table 1. There were no differences between two different sourced-origins in basic chemical profile ($P > 0.05$). The content of dry matter and ash was consistent with the results of the previous publications [15–17] and the tabular value of National Research Council [13], however, the content of ether extract in this study was much lower than that obtained by Yari et al. [17] and the tabular value of National Research Council [13], but is similar to the result of Mullins et al. [18]. The exact reason for lower in ether extract is not known, but the differences could be due to different origins and/or climate of growing season in each origin.

For forage protein profile, these two sourced-origins contained similar crude protein value (179 vs. 178 g/kg DM, $P > 0.05$). However, Bernard et al. [15] reported that the crude protein content of alfalfa hay (21% DM) was much higher than that obtained in this study, and however, Yari et al. [17] reported the crude protein content in alfalfa ranged from 17.7 to 20.4% DM which is in agreement with our results.

Comparing the two sourced-origins in other protein fraction profiles, there were no differences ($P > 0.05$) in non-protein nitrogen, soluble crude protein, and acid detergent insoluble crude protein contents, but the sourced-origins tended to influence the content of neutral detergent insoluble crude protein (113 vs. 155 g/kg CP, P = 0.059). The significant differences in neutral detergent insoluble crude protein between the sourced-origins are not known, but could be differences in nitrogen deposit in plant cell wall in different area with different soil and climate conditions. Lower content of acid detergent insoluble crude protein indicates its better protein quality [10,14], because acid detergent insoluble crude protein is negatively associated with forage quality.

The CSO tended to have better protein quality than the FSO due to lower content of acid detergent insoluble crude protein (unavailable protein: 59 vs. 68 g/kg CP). The non-protein nitrogen is the main fraction in soluble crude protein of modeled alfalfa hay, accounting for 66.9% of the soluble crude protein content. No differences were found

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