MNF Education

Plant-based food and feed protein structure changes induced by gene-transformation, heating and bio-ethanol processing: A synchrotron-based molecular structure and nutrition research program

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Received: April 16, 2010
Revised: June 2, 2010
Accepted: June 9, 2010

Unlike traditional ''wet'' analytical methods which during processing for analysis often result in destruction or alteration of the intrinsic protein structures, advanced synchrotron radiation-based Fourier transform infrared microspectroscopy has been developed as a rapid and nondestructive and bioanalytical technique. This cutting-edge synchrotron-based bioanalytical technology, taking advantages of synchrotron light brightness (million times brighter than sun), is capable of exploring the molecular chemistry or structure of a biological tissue without destruction inherent structures at ultra-spatial resolutions. In this article, a novel approach is introduced to show the potential of the advanced synchrotron-based analytical technology, which can be used to study plant-based food or feed protein molecular structure in relation to nutrient utilization and availability. Recent progress was reported on using synchrotron-based bioanalytical technique synchrotron radiation-based Fourier transform infrared microspectroscopy and diffused reflectance infrared Fourier transform spectroscopy to detect the effects of gene-transformation (Application 1), autoclaving (Application 2), and bio-ethanol processing (Application 3) on plant-based food and feed protein structure changes on a molecular basis. The synchrotron-based technology provides a new approach for plant-based protein structure research at ultra-spatial resolutions at cellular and molecular levels.

Keywords:
Gene-transformation / Heating and Bio-ethanol processing / Nutrient availability / Protein molecular structures / Synchrotron molecular spectroscopy

1 Introduction

To date, there has been little application of synchrotron radiation-based Fourier transform infrared microspectroscopy (SRFTIRM) to the study of molecular structures in plant-based food and feed protein in relation to nutrient availability [1]. Protein inherent structure, among other factors such as protein matrix, affects nutritive quality, fermentation, and degradation behavior in both humans and animals [2]. Protein structure profile influences protein value (*e.g.* absorbed protein in small intestine) and

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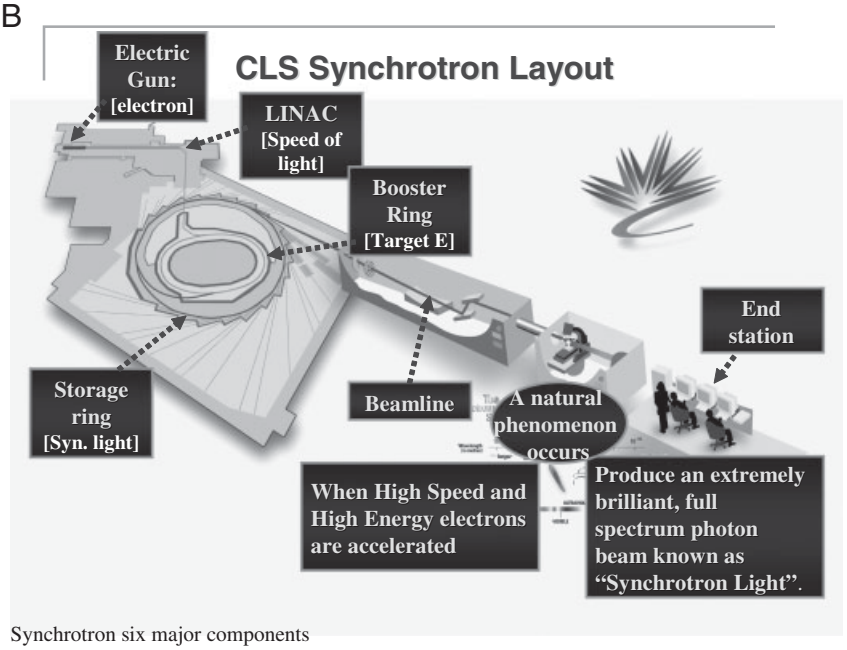
Abbreviations: AHCA, agglomerative hierarchical cluster analysis; **DRIFT,** diffused reflectance infrared Fourier transform; **PC,** principal component; **PCA,** principal component analysis; **SRFTIRM,** synchrotron radiation-based Fourier transform infrared microspectroscopy

A

Canadian Light Source (CLS, National Synchrotron Light Source)

B



Synchrotron six major components

Figure 1. Synchrotron six major components and Canadian Light Source (CLS) synchrotron layout. (A) Canadian Light Source (CLS, National Synchrotron Light Source). (B) Synchrotron six major components.

functionality (*e.g.* solubility) and affects the access of gastrointestinal digestive enzymes to the protein. Reduced accessibility results in poor digestibility and as a result, low-protein nutritive value [2, 3].

1.1 What is synchrotron and what are the major components?

A synchrotron is a giant particle accelerator that turns electrons into light (Fig. 1). It is composed of six major components: (i) electron gun, (ii) linear accelerator (also called LINAC, (iii) booster ring, (iv) storage ring, (v) beamlines, and (vi) end experimental stations [4] (CLS Synchrotron Facts: http://www.lightsource.ca/education/whatis.php). When high-speed and high-energy electrons are accelerated, it will produce extremely brilliant, full spectrum photon beam known as ''synchrotron light''. At the end of the experimental stations, researchers collect synchrotron-based data to determine molecular structure of a sample [1].

1.2 Advantage of bright synchrotron light

The synchrotron facility is able to produce extremely bright light, million times brighter than sunlight. The advantages of bright synchrotron light are shown in Fig. 2. The extremely bright synchrotron light makes it possible to detect biomaterial structure (chemical makeup) at both molecular and cellular levels [1, 5–7] (Miller, L. M. Infrared Microsperctroscopy and Imaging: http://nslsweb.nsls.bnl.gov/nsls/pubs/nslspubs/imaging0502/irxrayworkshopintroduction.ht).

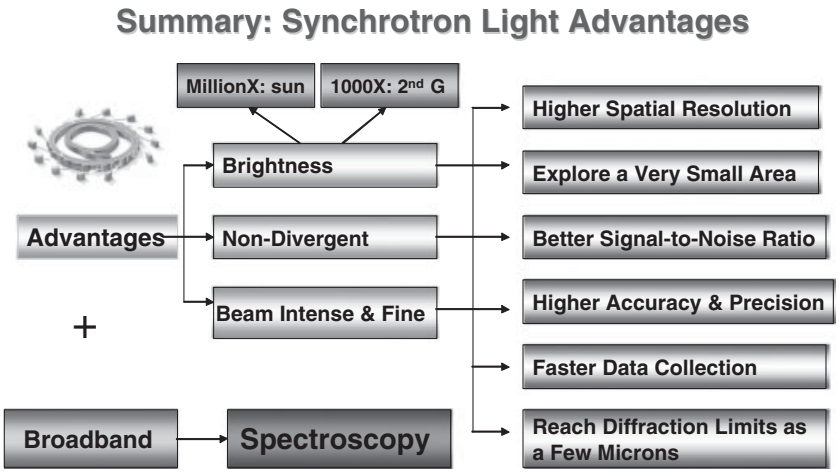


Figure 2. Synchrotron light advantages.

1.3 Why the need for advanced synchrotron-based bioanalytical technique (probe molecular structure)

Advanced SRFTIRM (Fig. 3) has been developed as a rapid, direct, nondestructive, and bioanalytical technique. In contrast to the traditional "wet" analytical (chemical) methods which, during processing for analysis, often result in destruction or alteration of the intrinsic structures (*e.g.* protein) of plants [8], this cutting-edge synchrotron-based analytical technology, taking advantages of synchrotron light brightness and small effective source size, is capable of exploring the molecular chemistry or structure of a biological tissue without destruction inherent structures at ultra-spatial resolutions [1, 5, 7, 9, 10].

1.4 The DRIFT molecular spectroscopy

The diffused reflectance infrared Fourier transform (DRIFT) spectroscopy is capable of exploring the biopolymer conformation through molecular and functional group spectral analyses [11, 12]. Using DRIFT, the diffusely reflected energy can be collected and a spectrum is produced, that is related to the sample chemical composition [13, 14]. Diffuse reflectance technique is usually utilized on samples that are powdered, or with irregular surfaces, or on nontransparent samples [14]. Therefore, DRIFT spectroscopy is particularly suitable for the examination of ground plant-based food and feed samples to reveal structural and chemical differences [15].

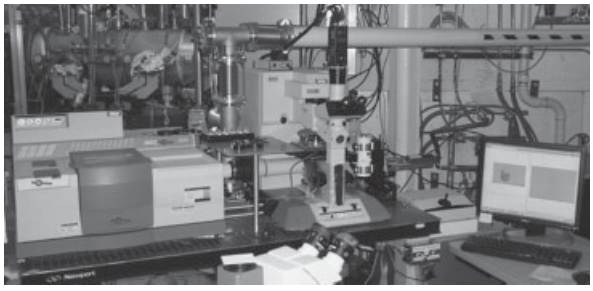
1.5 The objective of this article

In this article, a novel approach is introduced to show the potential of the advanced synchrotron-based bioanalytical technology – SRFTIRM, which can be used to study plant-based food and feed protein molecular structure and detect the effects of gene transformation [2], autoclaving [3, 12], and bio-ethanol processing [16] on plant protein structure changes on a molecular basis, in relation to protein nutrition value.

A

National Synchrotron Light Source (NSLS), Brookhaven National Lab (BNL)

B



Advanced synchrotron radiation-based infrared microspectroscopy (U2B station)

Figure 3. SRFTIRM (U2B station, National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, US Dept of Energy). (A) National Synchrotron Light Source (NSLS), Brookhaven National Lab (BNL). (B) Advanced SRFTIRM (U2B station).

2 Plant-based food and feed protein molecular structure and spectral features with SRFTIRM

2.1 Plant-based food and feed protein molecular structure

Plant protein has unique molecular structures; therefore, it has its own infrared spectrum [5, 17–19]. Plant protein infrared spectrum has two primary features: the amide I

frequency band (*ca.* 1600–1700 cm^{-1}) and amide II (*ca.* 1500–1560 cm^{-1}) frequency band (Fig. 4), both of which arise from specific stretching and bending vibrations within the protein backbone [5, 20, 21].

2.2 Protein amide I and amide II and α-helix and β-sheet structure

The amide I band arises predominantly from the stretching vibration of the amide C = O group (80%), in addition to a minor C–N stretching vibration [20, 21] and is particularly sensitive to changes in protein secondary structure [1, 5–7, 18, 19, 22–26] (Miller, L. M. Infrared Microsperctroscopy and Imaging.: http://nslsweb.nsls.bnl.gov/nsls/pubs/nslspubs/imaging0502/irxrayworkshopintroduction.ht) (Fig. 5). Changes in the amide I patterns can be used to predict protein secondary structure [27, 28] (Fig. 5). For example, variability in desiccation tolerance of maize embryos was detectable through variation in the amide I band [18]. For α-helices, the amide I frequency band is typically in the range of 1648–1658 cm^{-1}. For β-sheets, the peak can be found within the range of 1620–1640 cm^{-1} [5, 6, 20, 21]. The amide II band is predominantly an N–H bending vibration (60%) coupled to significant C–N stretching (40%) and is also used to assess protein conformation [20, 21]. However, utility of the amide II band for protein structure prediction is lower than the amide I band, since the former arises from complex vibrations involving multiple functional groups [5, 17, 20, 21, 27].

2.3 Molecular chemistry imaging and mapping

Synchrotron-based infrared microspectroscopy is able to image molecular chemistry of grains such as wheat and corn (cv. Pioneer 39P78) to reveal ultraspatial intensity and distribution of nutrient relation chemical functional groups in the tissue [29]. The grain tissue can imaged from the pericarp, seed coat, aleurone, and endosperm under peaks at 1510 cm^{-1} (aromatic compound), 1650 cm^{-1} (amide I), 1246 cm^{-1} (cellulosic material), *etc.* Figure 6 shows that with synchrotron FTIR microspectroscopy, the images of molecular chemistry could be generated [29, 30].

2.4 Molecular structure in relation to nutrient availability

The protein amide I and II and α-helix and β-sheet structure ratios have been studied in relation to protein profile, protein subfraction, digestibility, and total protein nutrient availability [3, 31]. Protein nutrient utilization and availability includes (i) protein degraded balance, (ii) truly absorbed and digested rumen bypass protein, (iii) truly absorbed and digested microbial synthesized protein, (iv) endogenous protein, and (v) total truly absorbed and digested protein in small intestine or metabolizable protein. The detailed relationship between protein structural profiles and nutrient availability is summarized in the following sections: Applications.

3 Multivariate plant protein molecular spectra analyses to discriminate and classify plant-based food and feed protein molecular structures

Two multivariate molecular spectral analyses can be used to discriminate and classify plant protein molecular structures and protein structure changes.

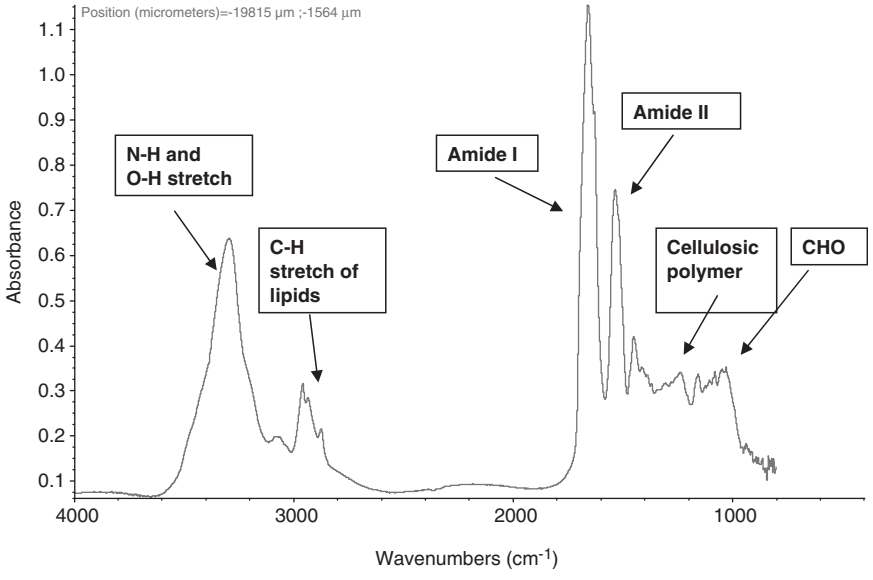


Figure 4. Typical plant-based food and feed protein spectrum within cellular and sub-cellular dimensions, showing protein amide I and amide II peaks, using SRFTIRM.

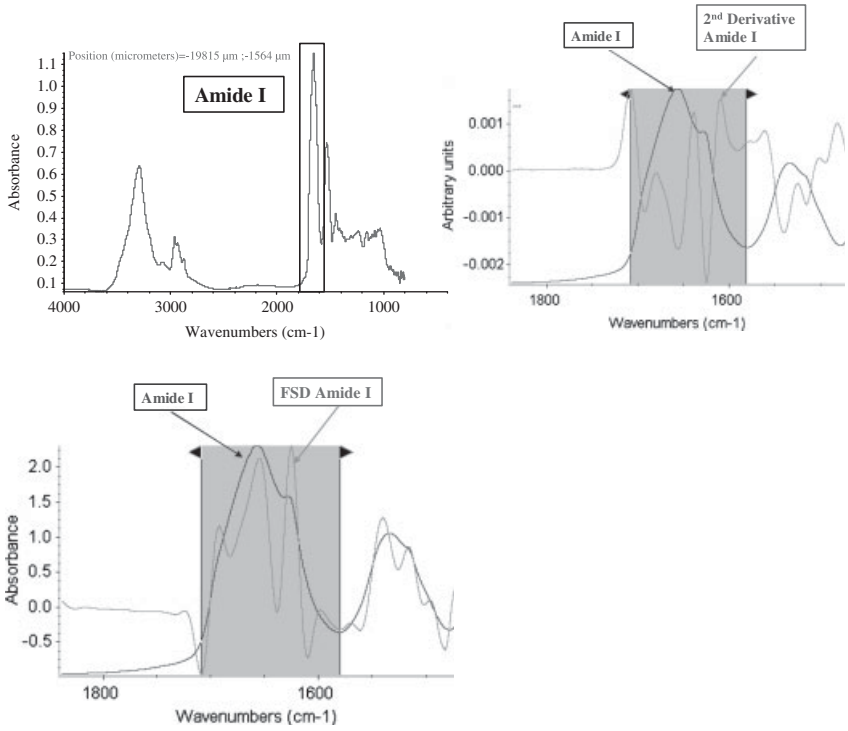


Figure 5. Plant-based food and feed protein amide I second derivative and FSD spectra, using SRFTIRM.

3.1 Agglomerative hierarchical molecular spectral cluster analysis

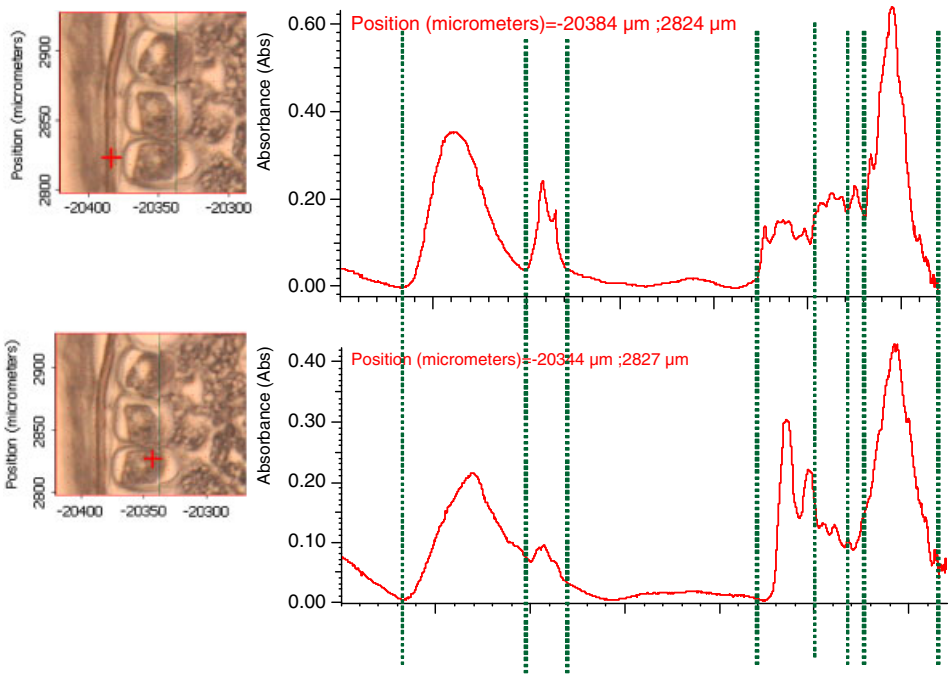
The first one is agglomerative hierarchical cluster analysis (AHCA). AHCA is initially used to try to determine the main sources of variation in SRFTIRM protein spectra and the results are displayed as dendrograms [31]. First, it calculates distance matrix, which contains information on the similarity of spectra. Then, in agglomerative hierarchical clustering, the algorithm searches within the distance matrix for the two most similar molecular spectra (minimal distance). There are several agglomerative hierarchical cluster distance methods for distance matrix calculation which includes (i) *D* values, (ii) Euclidean Distances, (iii) Normalized Euclidean Distances, (iv) Euclidean Squared Distances, and (v) City Block [31]. In the exampled studies, the Euclidean Distance was often used for distance matrix calculation. There are several cluster methods for clustering calculation which include (i) Average Linkage, (ii) Single Linkage, (iii) Complete Linkage, (iv) Group Average, (v) Centroid Method, (vi) Median Algorithm, and (vii) Wards Algorithm [31]. In the exampled studies, the Wards Algorithm was often used for clustering calculation. The equations for each cluster method are as follows. The detailed explanations and equations for each distance method and each cluster method are reported in Cytospec [31] and summarized by Yu [32]. Figure 7 shows that with AHCA molecular analysis, the raw and heated flaxseed (cv. Vimy) could be discriminated [12].

3.2 Principal components spectral cluster analysis

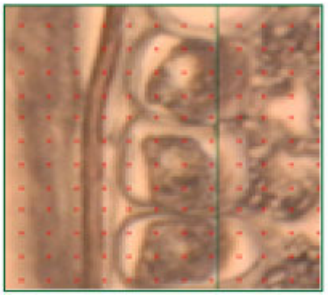
The second multivariate analysis used to determine major sources of variation in the SRFTIRM spectra is principal component analysis (PCA), called a statistical data reduction method that transforms the original set of variables to a new set of uncorrelated variables called principal components (PCs). The purpose of PCA is to derive a small number of independent linear combinations (PCs) for a set of variables, while retaining as much information in the original variables as possible. This analysis allows the global study of the relationships between a set of quantitative characters p (*e.g.* chemical functional groups) observed for a set of n samples (*e.g.* spectra). The basic idea in a multiple variable system is to extract, one, two, or sometimes more independent (orthogonal) PCs that carry maximum information. Typically, the first few PCs will account for $>95\%$ of the remaining observed variance. The outcome of such an analysis can be presented either as 2-D (two PCs) or 3-D (three PCs) scatter plots [33]. Figure 7 shows that with PCA molecular analysis, the raw and heated flaxseed (cv. Vimy) could be grouped separately.

3.3 Advantage of two molecular spectral analyses

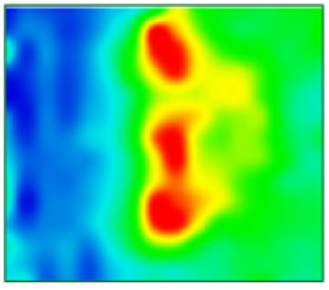
The big advantage of AHCA and PCA molecular spectral analyses is that we do not need to know what the spectral assignments are, since we just want to qualitatively separate one group from another.



Top: spectrum in seed coat tissue; Bottom: spectrum in aleurone cell



Visible image



Cell imaging protein to CHO ratio

Figure 6. Imaging molecular chemistry of wheat grain tissue, using SRFTIRM.

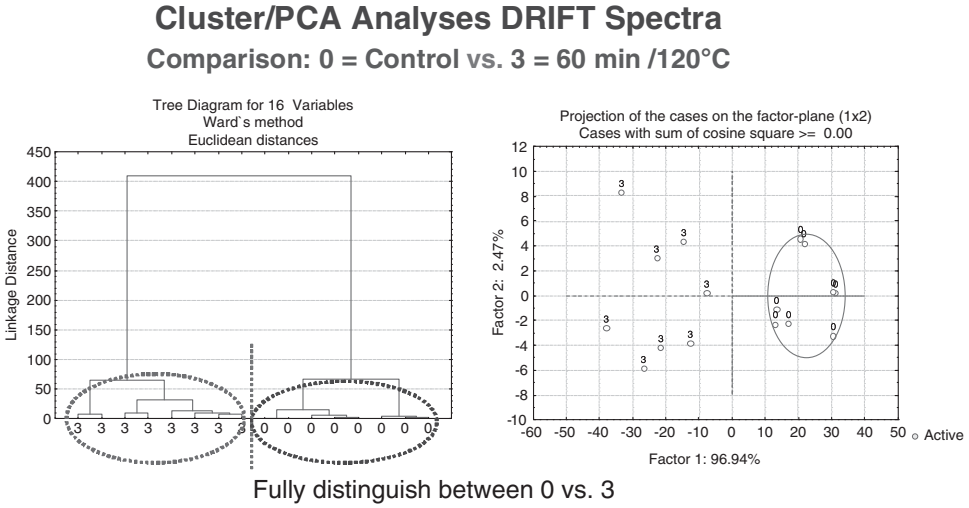


Figure 7. Using Cluster and PCA molecular spectral analyses of fingerprint region: 2000–800 cm^{-1} in the raw and autoclaving flaxseeds (cv. Vimy) (adapted from Doiron *et al.* [12]).

4 Plant-based food and feed protein structure changes on a molecular basis detected with SRFTIRM and DRIFT spectroscopy: Recent progress

4.1 Application I

Effect of proanthocyanidin and anthocyanin-enhanced *Lc*-gene transformation on molecular basis of protein structure changes in alfalfa plants.

This exampled study [2] aimed to use advanced synchrotron technique – SRFTIRM to compare protein molecular structure of alfalfa plant tissues transformed with the maize *Lc* regulatory gene with nontransgenic alfalfa protein within cellular and subcellular dimensions and to quantify protein inherent structure profiles using Gaussian and Lorentzian methods of multicomponent peak modeling [25].

Protein molecular structure revealed by this method included α-helices, β-sheets, and other structures such as β-turns and random coils. Hierarchical Cluster Analysis and PCA of the synchrotron data, as well as spectral analysis based on the curve fitting, showed that transgenic alfalfa contained a relatively lower ($p<0.05$) percentage of the model-fitted α-helices (29 *versus* 34) and model-fitted β-sheets (22 *versus* 27) and a higher ($p<0.05$) percentage of other model-fitted structures (49 *versus* 39). Transgenic alfalfa protein displayed no difference ($p>0.05$) in the ratio of α-helices to β-sheets (average: 1.4) and higher ($p<0.05$) ratios of α-helices to others (0.7 *versus* 0.9) and β-sheets to others (0.5 *versus* 0.8) than the nontransgenic alfalfa protein. The transgenic protein structures also exhibited no difference ($p>0.05$) in the vibrational intensity of protein amide I (average of 24) and amide II areas (average of 10) and their ratio (average of 2.4) compared with nontransgenic alfalfa.

Cluster analysis and PCA showed no significant differences between the two genotypes in the broad molecular fingerprint region, amides I and II regions, and the carbohydrate molecular region, indicating they are highly related to each other.

The results suggest that transgenic *Lc* alfalfa leaves contain similar proteins to nontransgenic alfalfa (because amide I and II intensities were identical), but subtle differences in protein molecular structure. Further study is needed to understand the relationship between these structural profiles and biological features such as protein nutrient availability, protein bypass, and digestive behavior of animals fed with this type of transgenic forage [2, 34].

4.2 Application II

Heat-induced plant protein structure and subfractions in relation to protein degradation kinetics and intestinal availability.

This exampled study [3, 12] aimed to reveal protein structures of flaxseed tissues affected by heat processing (Autoclaving) at a cellular level, using the advanced synchrotron technology as a novel approach, and quantify protein structure in relation to protein digestive kinetics and nutritive value [3, 12]. The parameters assessed included (i) protein structure α-helix to β-sheet ratio, (ii) protein subfractions profiles, (iii) protein degradation kinetics and effective degradability, and (iv) predicted nutrient supply in terms of the intestinally absorbed protein supply (DVE) and degraded protein balance (OEB). The experimental setup is shown in Fig. 8.

In this study [3], Vimy flaxseed protein was used as a model plant-based food/feed protein and autoclave-heated at 120°C for 20, 40, and 60 min as treatments T1, T2, and T3, respectively. The results showed that using the synchrotron-based bioanalytical technique – SFTIRM, the heat-induced protein structure changes were revealed and identified. The heating at 120°C for 40 and 60 min increased protein structure α-helix to β-sheet ratio. There were linear effects of heating time on ratio. The heating also changed protein chemical profiles, which showed soluble crude protein decreased on heating with

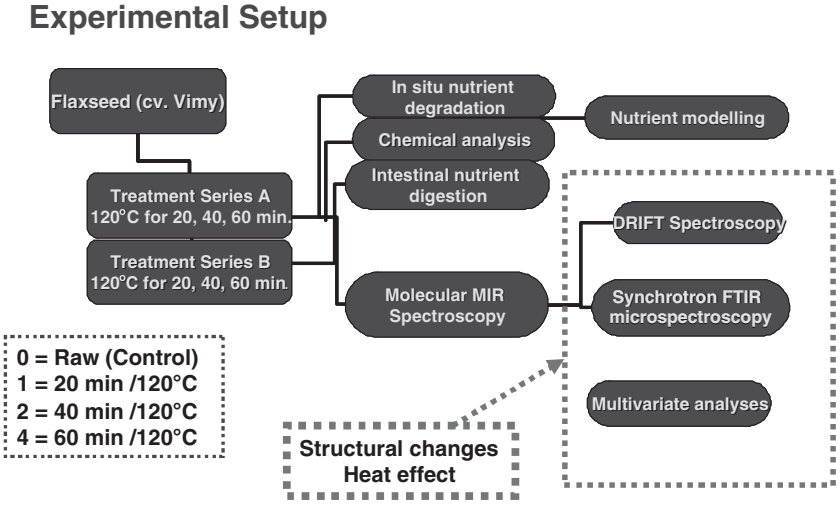


Figure 8. Experimental setup: Heat-induced plant-based food/feed protein structure in relation to protein degradation kinetics and intestinal protein availability.

concomitant increases in nonprotein nitrogen, neural, and acid detergent insoluble nitrogen. The protein subfractions with the greatest changes were PB1 (fraction PB1 is a rapidly degradable protein fraction) which showed a dramatic reduction, and PB2 (fraction PB2 is fermented at a lower rate than buffer-soluble fractions and some of the PB2 fraction escapes to the lower gut), showing a dramatic increase demonstrating a decrease in the overall protein degradability. *In situ* results showed a reduction in degradable protein without differences between the treatments. Protein intestinal digestibility by three-step *in vitro* showed no changes to undegradable protein. Modeling results showed that the heating increased total intestinally absorbable protein (DVE value) and decreased degraded protein balance (OEB value), but there were no differences between the treatments. There was linear effect of heating time on the DVE and cubic effect on the OEB value. The detailed correction results between the protein molecular structure and the protein utilization and availability are summarized in Table 1. The results showed that the heating changed protein chemical profiles, protein structure α-helix to β-sheet ratio, and protein sub-fractions, and decreased degradable protein and increased potentially nutrient supply to small intestines. The protein structure α-helix to β-sheet ratio had significantly positive correlation with total intestinally absorbed protein supply and negative correlation with degraded protein balance [3].

4.3 Application III

Effect of bio-ethanol processing on plant protein structure changes on a molecular basis: Study relationship of protein molecular structures to metabolic characteristics of the proteins in new co-products of bio-ethanol production.

This exampled study [16] aimed to reveal protein molecular structures of the new co-products of bio-ethanol production affected by bio-ethanol processing, identifying the differences in protein molecular structure between grains and new co-products and between different types of the bio-ethanol co-products and quantify protein molecular structures in relation to protein nutritive values. In this study, proteins were from wheat, corn, wheat DDGS, corn DDGS, and blend DDGS (wheat:corn = 70:30) from bio-ethanol production.

The results [16, 35] showed that the protein structure α-helix to β-sheet ratio was significantly different between the grains and the new co-products of bio-ethanol production. The α-helix to β-sheet ratio was significantly higher in

Table 1. Correlation between protein structure α-helix to β-sheet ratios and basic protein profiles, protein subfractions, *in situ* protein degradation kinetics, intestinal digestibility, and total protein nutrient availability in the raw (control) and autoclave treated Vimy flaxseed (Source from Doiron *et al.* [3] and used by permission of the *Journal of Dairy Science*)

Item	Correlation	
	Spearman correlation coefficient, *R*	*p*-Value
Basic protein profiles (% of DM)	with protein α-helix to β-sheet ratios	
Total crude protein content	−0.33	0.667
Soluble crude protein	−0.95	0.051
Neutral detergent insoluble protein	0.95	0.051
Acid detergent insoluble protein	0.95	0.051
Protein subfractions (% of CP)		
PA (nonprotein N with rapid degradation)	0.74	0.261
PB1 (fastly degraded protein)	−0.95	0.051
PB2 (intermediately degraded protein)	0.95	0.051
PB3 (slowly degraded protein fraction)	0.95	0.051
PC (unavailable protein)	0.95	0.051
In situ protein degradation kinetics (% of CP)		
Protein degradation rate (Kd), %/h	−0.83	0.167
Soluble protein fraction *in situ* (S)	−0.32	0.684
Undegradable protein fraction *in situ* (U)	0.95	0.051
Degradable protein fraction (D)	−0.74	0.261
Degradable protein content	−1.00	<0.001
Intestinal digestibility of the undegraded protein		
dRUP (%)	−0.63	0.368
Protein nutrient supply (g/kg DM)		
Absorbable microbial protein	−0.63	0.368
Endogenous protein losses	−0.95	0.051
Absorbable undegraded protein	0.95	0.051
Total intestinally absorbed protein supply (DVE)	0.95	0.051
Degraded protein balance (OEB)	−0.95	0.051

the grains than in the co-products (1.38 *versus* 1.03). There was significantly difference between wheat and corn (1.47 *versus* 1.29) but no difference between wheat DDGS and corn DDGS (1.04 *versus* 1.03). The protein structure amide I to II ratio was also significantly different between the grains and the new co-products. The amide I to II ratio was significantly higher in the grains than in the new co-products (4.58 *versus* 2.84). There were no significant differences between wheat and corn (4.61 *versus* 4.56) but significant differences between wheat DDGS and corn DDGS (3.08 *versus* 2.21). The above results indicated that bio-ethanol processing changes protein molecular structures, compared with original grains. The bio-ethanol processing also changed chemical profiles and protein subfraction profile. Bio-ethanol processing highly increased total intestinally absorbable protein. Both protein structure α-helix to β-sheet ratio and amide I to II ratio had significantly negative correlation with total intestinally absorbed protein supply and no significant correlation with degraded protein balance. Multiregression results show that the protein structure α-helix to β-sheet ratio was the most important parameter (among the amide I, amide II, amide I to II ratio, α-helix, β-sheet, α-helix to β-sheet ratio) and could be used to potential predict total intestinally absorbed protein supply ($R^2 = 0.89$) from the new co-products of bio-ethanol production. In summary, bio-ethanol processing changed protein molecular structure α-helix to β-sheet ratio and protein amide I to II ratio which highly related to nutrient values. The results indicated that the protein structure α-helix to β-sheet ratio in the new co-products of bio-ethanol productions is correlated to total intestinally absorbed protein supply [16]. Using spectral features of protein molecular structure as a predictor of the protein, nutrient availability is still in its preliminary stage. In order to obtain a more conclusive predictive equation, a large-scale *in vivo* study with various sources of bio-ethanol co-products is needed to test the applicability of the protein molecular structural parameters investigated [16].

5 Conclusions and implications

The above results demonstrate the potential of ultra-spatially resolved SRFTIRM to reveal plant-based food and feed protein structural differences due to genetic manipulation (gene transformation), autoclaving, and bio-ethanol processing. Figure 9 shows how to use SFTIRM and DRIFT molecular spectral spectroscopy for plant-based food molecular structure research. The multipeak modeling can be used to detect relative protein structure differences between the different treatments. Multivariate molecular spectral analyses can be used to qualitatively separate one group from another statistically. The results also show the relationship between plant-based food and feed protein molecular structure changes and nutritive availability. However, using spectral features of protein molecular structure to predict the protein nutrient availability is still in its preliminary stage, a validation is needed in a large-scale *in vivo* study.

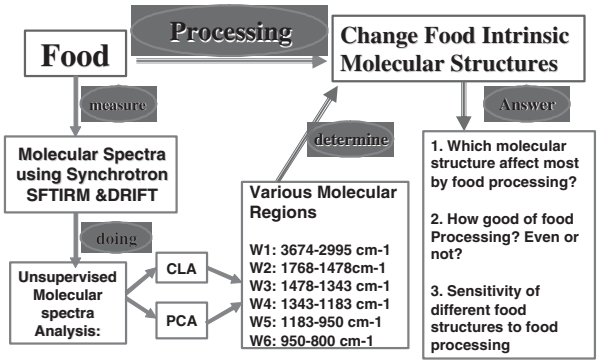


Figure 9. How to use SFTIRM and DRIFT molecular spectral spectroscopy for plant-based food molecular structure research.

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC – Individual Discovery Grant) and the Saskatchewan Agricultural Development Fund (ADF) and Ministry of Agriculture Strategic Research Chair Fund. The National Synchrotron Light Source in Brookhaven National Laboratory (NSLS-BNL, New York, USA) is supported by the US Department of Energy contract DE-AC02-98CH10886. The Center for Synchrotron Biosciences (U2B), Case Western Reserve University, was supported by the National Institute for Biomedical Imaging and Bioengineering under P41-EB-01979. The Canadian Light Sources (CLS) is supported by various federal and provincial funding agencies in Canada. The author is grateful to Dr. John McKinnon, Dr. Colleen Christensen, and Dr. David Christensen (University of Saskatchewan) for support and collaborations, and Megan Bourassa, Jennifer Bohon, Randy Smith, Nebojsa Marinkovic, Lisa Miller, Wang Qi, Alexander Ignatov, Randy Smith at U10B and U2B (National Synchrotron Light Source – Brookhaven National Laboratory (NSLS-BNL), US Department of Energy, Upton, New York), Robert Julian at Port 031 (SRC, University of Wisconsin, Madison) and Tim May, Tor Pederson and Luca Quaroni (Canadian Light Sources (CLS), University of Saskatchewan) for helpful data collection at 01B1-1 (Mid IR) experimental station.

The author has declared no conflict of interest.

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