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Relationship of protein molecular structure to metabolisable proteins in different types of dried distillers grains with solubles: a novel approach

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To date, there has been no study of protein molecular structures affected by bioethanol processing in relation to protein nutritive values of the new co-products of bioethanol production. The objective of the present study was to investigate the relationship between protein molecular structures (in terms of protein α-helix and β-sheet spectral intensity and their ratio and amide I to amide II spectral intensity and their ratio) and protein rumen degradation kinetics (rate and extent), estimated protein intestinal digestibility and total truly absorbed protein in small intestine (metabolisable protein) in different types of dried distillers grains with solubles (DDGS), such as wheat DDGS, maize DDGS and blend DDGS (wheat:maize = 70:30). The protein molecular structures of the different types of DDGS affected by processing were identified using diffuse reflectance IR Fourier transform spectroscopy. The results showed that the protein structure α-helix to β-sheet ratio in the DDGS had a strongly negative correlation with estimated intestinal digestibility of ruminally undegraded protein (%dRUP, R −0·95, P=0·04), tended to have a significant correlation with the protein PC subfraction (which was undegradable and contained proteins associated with lignin and tannins and heat-damaged proteins) (R 0·91, P=0·09) and had no correlation (P>0·10) with rumen degradation kinetics (rate and extent), total intestinally absorbed protein supply and degraded protein balance. However, the protein amide I to amide II ratio in the DDGS had a strongly positive correlation with soluble crude protein (CP) (R 0·99, P<0·01), protein PA subfraction (which was instantaneously solubilised at time zero) (R 0·99, P<0·01), protein PB2 subfraction (which was intermediately degradable) (R −0·95, P=0·04) and total digestible CP (R 0·95, P=0·04). The amide I to amide II ratio also had strongly negative correlations with ruminally undegraded protein (%RUP: R −0·96, P=0·03) and the degraded protein balance (OEB: R −0·97, P=0·02), but had no correlation (P>0·10) with the total intestinally absorbed protein supply. Multiple regression results show that the protein structure α-helix to β-sheet ratio was a better predictor of %dRUP with R^2 0·92. The amide I to II ratio was a better predictor of the degraded protein balance with R^2 0·93 in the DDGS. In conclusion, the changes in the protein molecular structure α-helix to β-sheet ratio and the amide I to amide II ratio during bioethanol processing (either due to fermentation processing or due to heat drying) were highly associated with estimated protein intestinal digestibility and degraded protein balance, but were not associated with total intestinally absorbed protein supply from the DDGS to dairy cattle. The present study indicates that a potential novel method could be developed based on the protein molecular structure parameters to improve the estimation of protein value after a validation in a large-scale *in vivo* study is done.

Protein molecular structures: α-Helix to β-sheet ratio: Amide I to amide II ratio: Bioethanol co-products: Metabolisable proteins

The utilisation and availability of proteins depend on the types of proteins and their specific susceptibility to enzymatic hydrolysis in the gastrointestines, and are also highly associated with protein molecular structures and profiles[1,2]. Protein secondary structures include mainly α-helix and β-sheet, and small amount of β-turn and random coil[3–6]. The protein molecular structure profiles (such as mid-IR molecular absorption intensities and ratios of these secondary structures) and molecular spectral characteristics of protein structure amide I and amide II and their ratio may influence protein quality, nutrient utilisation, availability and digestive behaviour in both animals and human subjects[7–10], mainly because protein structure affects access to microbial degradation and gastrointestinal digestive enzymes, which affects protein values and protein availability (total intestinally absorbed protein supply).

However, studies on protein structures at molecular and cellular levels in relation to nutrient availability and digestive behaviours of proteins are still limited compared with traditional animal nutrition research. The protein molecular structure profile could be significantly affected by heat (autoclaving) processing[2] and gene transformation[11].

Recently, the different types of new co-products from bioethanol processing, wheat dried distillers grains with solubles (wheat DDGS), triticale DDGS, pea DDGS, hull-less barley DDGS, maize DDGS and blend DDGS (e.g. wheat:maize = 70:30; 50:50; 20:80), were produced in North America[12]. The variation in nutrient profiles between and within DDGS types is attributed to grain type, grain varieties, processing methods and/or processing conditions. During bioethanol processing, ethanol fermentation removes most of the starch from grain kernels and concentrates the

Abbreviations: DDGS, dried distillers grains with solubles; CP, crude protein; NRC, National Research Council; RUP, ruminally undegraded protein.

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other components, mainly protein, fibre, fat and minerals in DDGS. Additionally, the DDGS are heat dried, which affects the protein molecular structure. So far, none of the published studies in the literature reported how the molecular structure changes in the DDGS are associated with nutrient availability in the rumen and intestine in dairy cattle. None of the published studies in the literature reported the possibility of using the protein molecular structure profile as a predictor of the nutrient value of the co-products. The DDGS can be a major source of ruminally undegraded protein (RUP) in diets, but it can vary greatly[12]. The protein molecular structure profiles might account for some of this large variation and would improve the estimation of protein value of different types of DDGS.

The objectives of this novel study were to investigate the relationship between protein molecular structures (in terms of protein α-helix and β-sheet intensity and their ratio and amide I to amide II intensity and their ratio) and protein rumen degradation kinetics (rate and extent), estimated protein intestinal digestibility, degraded protein balance[13] and total truly absorbed protein in small intestine (metabolisable protein)[13] in different types of DDGS. The hypothesis of this preliminary study was that the differences in protein molecular structures in bioethanol co-products were associated with the various measures of protein degradability and digestibility, which could be used as predictors of protein nutrient availability.

Materials and methods

The experiments were carried out at the Saskatchewan Structure Sciences Center and Livestock Research Station at the University of Saskatchewan.

The animal experiment (protocol no. 19910012) was reviewed and approved by the Animal Care Committee of the University of Saskatchewan, and was conducted in accordance with the guidelines of the Canadian Council on Animal Care[14].

Co-products of bioethanol production, original cereal grains and nutrient analysis

The different types of bioethanol co-products, wheat DDGS, maize DDGS and blend DDGS (wheat:maize = 70:30), and original feedstock wheat and maize samples from bioethanol plants were used for the present study. The detailed sampling structure and collection time frames were described previously in the publication by Nuez-Ortín & Yu[12] in 2009. The detailed methods and calculations for chemical and nutrient analyses[15–19], Cornell net carbohydrate and protein system (CNCPS) protein fractionation[20,21], *in situ* rumen degradation[22–24], estimated intestinal protein digestion[25] and modelling total nutrient supply[26,27], in dairy cattle fed the bioethanol co-products were reported by Nuez-Ortín & Yu in 2010[28,29]. In the present study, the chemical, nutrient and structure profile data of the six different types of DDGS and original grain samples (summarised in Table 1)[12,28,29] were used in a correlation study between protein molecular structure profiles (amide I, amide II, amide I to amide II ratio, α-helix, β-sheet and α-helix to β-sheet ratio) and nutrient availability.

Diffuse reflectance IR Fourier transform spectroscopy

The DDGS samples were finely ground two times to pass through a 250 μm screen (Retsch ZM-1, Brinkmann Instruments (Canada) Limited, ON, Canada). Samples of the ground DDGS were then mixed with potassium bromide (IR grade; P5510, Sigma, St Louis, MO, USA) in a ratio of four parts co-product to one part potassium bromide in a 2 ml centrifuge tube and mixed by vortexing for several minutes. Diffuse reflectance IR Fourier transform spectroscopy was performed using a Bio-Rad FTS-40 with a ceramic IR source and mercury-cadmium-telluride (MCT) detector (Bio-Rad laboratories, Hercules, CA, USA). Data were collected using Win-IR software (Bio-Rad Digilab, Cambridge, MA, USA). Spectra were generated from the mid-IR (4000–800 per cm) portion of the electromagnetic spectrum with 256 co-added scans and a spectral resolution of 4 per cm[2].

Molecular spectral analysis of protein amide I and amide II ratio and α-helix and β-sheet ratio

Molecular spectral analysis was done with OMNIC 7.2 software (Spectra Tech, Madison, WI, USA). Chemical functional groups in the grains and DDGS (protein amide I and amide II and protein secondary structures α-helix and β-sheet) were identified according to the published reports[6,7,30–32]. The protein IR spectrum has two primary features. The protein amide I bond is primarily C═O stretching vibration (80 %) plus C—N stretching vibration[5,33]. Protein amide I absorbs at approximately 1655 per cm. Protein amide II which absorbs at approximately 1550 per cm consists primarily of N—H bending vibrations (60 %) coupled with C—N stretching vibrations (40 %)[5,33]. The absorption intensity of peak area of amide I and amide II and their ratio were calculated with baseline region at approximately 1720–1485 per cm. The vibrational frequency of the protein amide I band is particularly sensitive to protein secondary structure[3,6,7,34,35]. Protein α-helix is typically in the range of approximately 1648–1660 per cm and β-sheet is in the range of approximately 1625–1640 per cm. To estimate the intensity of α-helix and β-sheet, two steps were applied. The first step used Fourier self-deconvolution (a method for resolving intrinsically overlapped bands) and the 2nd derivative function in OMNIC 7.2 to obtain the Fourier self-deconvolution and 2nd derivative spectrum in protein amide I region at approximately 1720–1575 per cm only to identify protein amide I component peak frequencies. The detailed concepts and algorithm of Fourier self-deconvolution were described by Kauppinen *et al.*[36] and Griffiths & Pariente[37]. The second step was done to quantify the intensity of peak height of α-helix and β-sheet and their ratio[2].

Statistical analysis

Correlation analysis. The relationship between the changes in protein structure profiles (in terms of α-helix to β-sheet ratio and amide I to amide II ratio) and changes in chemical and nutrient profiles (in terms of chemical composition, protein fractions, *in situ* rumen degradation kinetics, estimated intestinal digestion and predicted nutrient supply to cattle) in the different types of DDGS samples (no original grain data)

Table 1. Summary of chemical, nutrient and protein molecular structure profiles of different grains (wheat, maize) and different types of dried distillers grains with solubles (DDGS) (wheat DDGS, maize DDGS and blend DDGS (wheat:maize = 70:30) with total sample number *n* 6) from bioethanol production* (data sources [12,28,29])

	Grains		Bio-ethanol co-products			Variation	
Item	Wheat	Maize	Wheat DDGS	Maize DDGS	Blend DDGS	SD	Approximate range difference from all data
Basic chemical composition							
DM (%)	89·2	88·7	92·2	90·9	91·8	1·6	3·5
Ash (% of DM)	2·0	1·9	5·2	4·2	5·3	1·7	3·4
OM (% of DM)	99·0	98·1	94·8	95·8	94·7	2·0	4·3
Ether extract (% of DM)	1·9	5·5	5·6	16·3	8·7	5·4	14·4
Structural and non-structural CHO profiles							
Total CHO (% of DM)†	81·0	82·1	48·9	46·6	49·1	18·3	35·5
Starch (% of DM)	60·9	68·7	6·4	4·2	2·7	33·2	66
Non-fibre CHO (% of CHO)‡	81·5	81·0	48·3	13·6	35·4	29·5	67·9
NDF (% of DM)	17·4	16·2	48·2	51·6	50·4	18·3	35·4
ADF (% of DM)	3·7	4·2	10·4	15·2	12·3	5·1	11·5
Acid-detergent lignin (% of DM)	1·1	0·7	3·4	2·2	3·6	1·3	2·9
Hemicellulose (% of DM)§	13·7	12·0	37·7	36·4	38·0	13·5	26
Cellulose (% of DM)‖	2·6	3·5	7·0	13·0	8·7	4·2	10·4
Protein profiles							
Total CP (% of DM)	15·0	10·5	40·2	32·8	36·9	13·4	29·7
SCP (% of CP)	21·5	70·7	15·3	10·5	14·5	25·0	60·2
NPN (% of SCP)	99·8	26·5	100·0	100·0	100·0	32·8	73·5
Acid-detergent insoluble protein (% of CP)	0·0	0·1	4·2	3·9	1·8	2·0	4·2
Neutral-detergent insoluble protein (% of CP)	16·3	5·4	56·8	34·5	50·6	21·9	51·4
Protein subfractions (CNCPS)¶							
PA (% of CP)	21·5	18·7	15·3	10·5	14·5	4·2	11
PB1 (% of CP)	0·0	52·0	0·0	0·0	0·0	23·3	52
PB2 (% of CP)	62·2	23·9	27·9	55·0	34·9	16·9	38·3
PB3 (% of CP)	16·3	5·3	52·6	30·7	48·8	20·4	47·3
PC (% of CP)	0·0	0·1	4·2	3·9	1·8	2·0	4·2
True protein (% of CP)**	78·5	81·2	80·6	85·7	83·7	2·8	7·2
PB1 (% of true protein)	0·1	64·0	0·0	0·0	0·0	28·6	64
PB2 (% of true protein)	79·3	29·5	34·9	64·2	41·7	21·1	49·8
PB3 (% of true protein)	20·7	6·6	65·1	35·8	58·3	24·7	58·5
Protein molecular structure profiles (unit: IR absorbance intensity)							
Amide I	162·45	64·42	291·79	261·81	274·68	96·19	227·37
Amide II	35·14	14·15	95·03	118·45	92·03	44·13	104·30
Amide I to amide II ratio	4·61	4·56	3·08	2·21	2·97	1·06	2·40
Protein α-helix	2·25	0·9	3·38	3·25	3·02	1·03	2·48
Protein β-sheet	1·53	0·7	3·22	3·14	3·02	1·14	2·52
α-Helix to β-sheet	1·47	1·29	1·04	1·03	0·99	0·21	0·48
Total digestible nutrients							
$TDN_{1\times}$ (% of DM)	83·4	88·7	78·2	90·5	80·1	5·3	12·3
Energy values (NRC-2001, NRC-1996 summary approach)							
$NE_{L3\times}$-dairy, MJ/kg DM (Mcal/kg DM)	7·95 (1·90)	8·50 (2·03)	8·46 (2·02)	9·92 (2·37)	8·62 (2·06)	0·73 (0·18)	1·97 (0·47)
NEm-beef, MJ/kg DM (Mcal/kg DM)	8·62 (2·06)	9·08 (2·17)	9·00 (2·15)	10·13 (2·42)	9·08 (2·17)	0·56 (0·13)	1·51 (0·36)
NEg-beef, MJ/kg DM (Mcal/kg DM)	5·86 (1·40)	6·24 (1·49)	6·15 (1·47)	7·12 (1·70)	6·24 (1·49)	0·47 (0·11)	1·26 (0·30)

Table 1. *Continued*

Item	Grains		Bio-ethanol co-products			Variation	
	Wheat	Maize	Wheat DDGS	Maize DDGS	Blend DDGS	SD	Approximate range difference from all data
In situ rumen degradation kinetics							
Soluble fraction *in situ* (% of CP)	3·7	1·6	9·8	3·8	7·3	3·3	8·2
Degradable fraction (% of CP)	90·1	97·1	90·2	71·2	89·4	9·7	25·9
Undegradable fraction (% of CP)	6·3	1·4	0·0	25·0	3·3	10·2	25
Degradation rate (%/h)	16·6	3·2	4·2	4·3	4·6	5·6	13·4
% RUP	30·2	64·7	53·4	66·5	54·1	14·5	36·3
RUP (g/kg DM)	53	79	228	230	212	87	177
Intestinal							
dRUP (%)	78	70	86	85	92	8·4	22
Predicted nutrient supply (DVE/OEB model)							
Fermentable OM (g/kg DM)	760	517	586	514	570	100·5	246
Absorbable microbial CP (g/kg DM)	73	49	56	49	54	9·9	24
Absorbable ruminally bypassed CP (g/kg DM)	46	62	218	216	216	89·3	172
ENDP (g/kg DM)	5	2	7	5	7	2·0	5
Total intestinally absorbed protein supply (DVE, g/kg DM)	114	109	267	261	263	83·4	158
Degraded protein balance (DVE/OEB model)							
Degraded protein balance (OEB, g/kg DM)	3	-43	86	14	72	52·8	129
Predicted nutrient supply (NRC-2001 model)							
Absorbable microbial CP (g/kg DM)	64	23	60	63	61	17	41
Absorbable ruminally undegraded CP (g/kg DM)	42	55	196	195	194	80	154
Total metabolisable protein supply (g/kg DM)	109	83	260	262	260	91	180

OM, organic matter; CHO, carbohydrate; NDF, neutral-detergent fibre; ADF, acid-detergent fibre; CP, crude protein; SCP, soluble crude protein; NPN, non-protein nitrogen; CNCPS, Cornell net carbohydrate and protein system; TDN, total digestible nutrient; NRC, National Research Council; NE_{L3X}, net energy for lactation at production level of intake (3x); NE_m, net energy for maintenance; NE_g, net energy for growth; RUP, ruminally undegraded protein; dRUP, digestibility of RUP; DVE, truly digested protein in the small intestine; OEB, degraded protein balance; ENDP, endogenous CP losses; EE, ether extract; NDIP, neutral-detergent insoluble protein; ADL, acid-detergent lignin.
Range indicates the range in all data, not just treatment means.
* These data were used to study the relationship between protein molecular structures and protein availability.
† Carbohydrate was calculated as: carbohydrate = 100 − EE − CP − ash[19].
‡ Non-fibre carbohydrate = 100 − (NDF − NDIP) − EE − CP − ash[19].
§ Hemicellulose = NDF − ADF[19].
‖ Cellulose = ADF − ADL[19].
¶ Protein subfractions using CNCPS include PA = fraction of CP that is instantaneously solubilised at time zero; PB1 = fraction of CP that is soluble in borate-phosphate buffer and precipitated with TCA; PB2 = calculated as total CP minus the sum of fractions PA, PB1, PB3 and PC; PB3 = calculated as the difference between the portions of total CP recovered with NDF and ADF; PC = fraction of CP recovered with ADF, and is considered to be undegradable. It contained proteins associated with lignin and tannins and heat-damaged proteins such as the Maillard reaction products.
** True protein = PB1 (% of CP) + PB2 (% of CP) + PB3 (% of CP).

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were analysed using the CORR procedure of Statistical Analysis Systems (SAS version 9.1.3, SAS Institute Inc., Cary, NC, USA) with the FISHER option, which offers confidence limits and *P*-values for Pearson's correlation coefficients based on Fisher's *z* transformation. The normality tests were performed using UNIVARIATE procedure of SAS with NORMAL and PLOT options.

Multiple regression analysis

In order to determine which protein molecular structure parameters (amide I to amide II ratio and α-helix to β-sheet ratio) in the different types of DDGS samples (no original grain data) play an important role in determining protein utilisation and availability to cattle, a multiple regression analysis was carried out using the 'PROC REG' procedure of SAS (version 9.1.3) with a model as follows:

Model: Y = amide I to amide II ratio (R_I-II) + α-helix to β-sheet ratio (R_α-β).

The model variable selection used a 'STEPWISE' option with variable selection criteria: 'SLENTRY = 0·05, SLSTAY = 0·05'. All variables left in the final model are significant at the 0·05 level. The residual analysis was carried out to test the regression model assumptions using UNIVARIATE procedure of SAS with NORMAL and PLOT options.

For all statistical analyses, significance was declared at $P<0·05$ and trends (tendency) at $P<0·10$. Differences among the treatments were evaluated using a multiple comparison test following Fisher's protected least significant difference method.

Results and discussion

The traditional approach to study protein value of different types of co-products from bioethanol production is to focus on total protein composition or total available protein composition using conventional 'wet' chemical analysis as suggested by National Research Council (NRC)-2001, which is called 'a chemical summary approach'. However, using the NRC-2001 summary approach to determine the protein value of bioethanol co-products is not accurate. A fatal shortcoming of the NRC-2001 summary is that it does not consider the intrinsic structure and molecular make-up of the different types of co-products. Traditional 'wet' chemical analysis can determine total protein composition, but fails to detect protein inherent structure and molecular make-up due to destruction of the bioethanol co-product protein structure during the processing for chemical analysis. In a previous research, it was found that two barley varieties showed the same chemical composition, but significantly different digestive behaviour in ruminants. The two co-products had similar levels of RUP, but different availability of RUP. The NRC-2001 summary approach cannot address these issues. Therefore, a new approach that is able to account for structural variation at molecular and cellular levels between the different types of co-products from bioethanol production should be developed. This novel approach should provide a new concept and methodology for true protein value research. Our new approach in this preliminary study was to look at the protein molecular structure (amide I, amide II, α-helix and β-sheet structure profiles) in the different types of bioethanol co-products in relation to true protein value.

Protein structure amides I and II profiles in relation to nutrient profiles in bioethanol co-products

The amide I and amide II structure profiles depend on the protein molecular structural make-up, and can be affected by cereal grain variety[38], autoclave-heated processing conditions[2] and gene transformation[11]. Bioethanol processing involves enzymatic fermentation and heat drying of the DDGS products, which affects molecular structural characteristics of protein amide I and amide II and their ratios[29]. The differences in the amide I to amide II profiles and their ratio may be associated with differences in nutrient value. However, no study has been reported. Table 2 shows the correlations of protein structure amide I, amide II and amide I to amide II ratio with nutrient profiles in the DDGS. There were significantly positive correlations between protein structure amide I and amide II ratio and soluble crude protein (CP) with R 0·99 ($P<0·01$), and a tended positive correlation with neutral-detergent insoluble protein with R 0·93 ($P=0·07$), but no correlation ($P>0·10$) with acid-detergent insoluble protein (Table 2). These results indicate that higher protein amide I and amide II ratio associated with higher neutral-detergent insoluble protein in the bioethanol co-products. For Cornell net carbohydrate and protein system (CNCPS) protein subfraction correlation, the results showed that the protein amide I and amide II ratio had strongly positive correlations with the protein PA fraction ($P<0·01$) with R 0·99 and a negative correlation with the protein PB2 subfraction ($P=0·04$) with R −0·95, but that it had no correlation ($P>0·10$) with the PB1, PB3 and PC fractions. For total digestible CP, the results showed that the protein amide I and amide II ratio had strongly positive correlation with total digestible CP (R 0·95, $P=0·05$). (For explanation of protein subfractions, see Table 1.)

For the correlation of *in situ* degradation parameters, the results showed that the protein amide I and amide II ratio had a correlation with the soluble fraction (R 0·94, $P=0·06$), potentially degradation fraction (R 0·99, $P<0·01$), undegradable fraction (R −0·99, $P<0·01$) and RUP (−0·96, $P=0·03$), but that it had no correlation with degradation rate ($P>0·10$). The result indicated that lower protein amide I and amide II ratio was associated with a higher RUP value in DDGS. The protein amide I and amide II ratio had no correlation with estimated intestinal digestibility of RUP *in vitro* ($P>0·10$). For predicted nutrient supply correlation, the results showed that the protein amide I and amide II ratio had no correlation ($P>0·10$) with microbial protein synthesis absorbable microbial CP, endogenous CP losses, absorbable ruminally undegraded CP and total intestinally absorbed protein supply, but that it had strongly positive correlations with the rumen degraded protein balance (R 0·97, $P=0·02$).

In a previous study, Doiron *et al.*[2] reported that heating the Vimy flaxseed changed the chemical profiles, which decreased the soluble CP upon heating with a concomitant increase in non-protein N, neutral-detergent insoluble nitrogen and acid-detergent insoluble nitrogen. They found that the protein subfractions with the greatest changes were PB1 (fraction PB1 is a rapidly degradable protein fraction in the rumen), which showed a dramatic reduction, and PB2 (fraction PB2 is fermented in the rumen at a lower rate than buffer-soluble fractions and some of the PB2 fraction escapes to the lower gut), which showed a dramatic increase, demonstrating

Table 2. Correlation between protein structures (amide I to amide II and their ratio) and chemical profiles, Cornell net carbohydrate and protein system (CNCPS) protein fractions, *in situ* degradation kinetics and nutrient supply in the different types of co-products (wheat dried distillers grains with solubles (wheat DDGS), maize DDGS and blend DDGS with total sample number *n* 4) from bioethanol production (include just dependent variables that were significantly affected by the ratios)

	Protein molecular structure (amide I, amide II and their ratio)					
	Amide I		Amide II		Ratio of amide I to amide II	
Items	Correlation coefficient (*R*)	*P*	Correlation coefficient (*R*)	*P*	Correlation coefficient (*R*)	*P*
Protein profiles						
CP (% of DM)	0·32	0·68	−0·25	0·74	0·94	0·06
Soluble crude protein (% of CP)	0·37	0·63	−0·24	0·75	0·99	<0·01
NDIP (% of CP)	−0·09	0·91	−0·65	0·35	0·93	0·07
Protein subfractions using the CNCPS system*						
PA (% of CP)	0·37	0·63	−0·24	0·75	0·99	<0·01
PB2 (% of CP)	0·01	0·98	0·59	0·41	−0·95	0·04
PB2 (% of true protein)	0·13	0·87	0·68	0·32	−0·92	0·08
PB3 (% of true protein)	−0·13	0·87	−0·68	0·32	−0·92	0·08
Digestible nutrients (NRC-2001 summary approach)						
Total digestible CP (% of DM)	0·19	0·80	−0·38	0·62	0·95	0·04
In situ rumen degradation kinetics						
Soluble fraction *in situ* (% of CP)	0·08	0·92	−0·48	0·52	0·94	0·06
Degradable fraction (% of CP)	0·19	0·80	−0·42	0·58	0·99	<0·01
Undegradable fraction (% of CP)	−0·17	0·83	0·44	0·55	−0·99	<0·01
%RUP	0·48	0·52	0·13	0·86	−0·96	0·03
Degraded protein balance (DVE/OEB model)						
Degraded protein balance (OEB, g/kg DM)	0·43	0·56	−0·17	0·82	0·97	0·02
Predicted nutrient supply (NRC-2001 model)						
Absorbable microbial CP (g/kg DM)	−0·29	0·70	0·28	0·72	−0·95	0·05

CP, crude protein; NDIP, neutral-detergent insoluble protein; NRC, National Research Council; DVE, truly digested protein in the small intestine; OEB, degraded protein balance.
* For explanation of protein subfractions, see Table 1.

a decrease in the overall protein degradability. Their *in situ* results showed a reduction in rumen-degradable protein, but the intestinal digestibility analysed by a three-step *in vitro* method showed no changes in RUP. Modelling results showed that the heating increased total intestinally absorbable protein (feed DVE value) and decreased the degraded protein balance (feed OEB value), but that there were no differences between the treatments. There was a linear effect of heating time on the DVE and cubic effect on the OEB value. However, in their study[2], no correlation study was done between amide I and amide II profiles and nutrient availability in Vimy flaxseed autoclaved at various conditions. No comparison could be made with the present study on the co-products of bioethanol processing.

Protein secondary structure profiles in relation to nutrient profiles in bioethanol co-products

The amide I band vibrational frequency can be used to determine the secondary structure of proteins because it is particularly sensitive to protein secondary structure[7,33,34,39]. For the protein α-helix structure, the amide I typically is in the range of approximately 1648–1660 per cm. For β-sheet, the peak is in the range of approximately 1620–1640 per cm[6,34]. The amide II can also be used to assess the protein conformation and protein molecular chemical make-up. However, it arises from complex vibrations involving multiple functional groups, and it is less useful for protein structure prediction than the protein amide I band[33]. In the DDGS, the spectrum of protein amide I original band shows peak centres at approximately 1658 and 1628 per cm. This was confirmed from both the Fourier self-deconvolution spectrum of amide I and the 2nd derivative spectrum of amide I at the region of approximately 1720–1575 per cm.

The DDGS differed in protein secondary structure conformation in terms of the ratio of protein α-helix and β-sheet, indicating the differences in protein molecular structural make-up and features. Bioethanol processing may change the protein molecular structure of the DDGS compared with the original products. These structural differences may impact the DDGS protein utilisation and availability in the rumen and intestine in ruminants.

Some microbial CP (such as yeast) that are present in the DDGS might affect the protein structure profiles of the DDGS, although the amount of microbial CP in the products is relatively small. During heat drying process, heat also denatures yeast, rendering them resistant to rumen degradation. While some of the protein content in the solubles is heated yeast[40,41], only 20 % of them are ruminally degradable[42]. Additionally, the solubles contribute to the RUP and absorbable ruminally undegraded CP contents in DDGS by providing simple sugars that increase the susceptibility to the Maillard reaction during heat drying[28]. Dorion *et al.*[2] reported that using the synchrotron radiation-based Fourier transformed IR microspectroscopy, and heating at 120°C for 40 and 60 min (not for 20 min) increased the protein structure α-helix to β-sheet ratio of Vimy flaxseed. There were linear effects of heating time on the ratio.

Table 3 shows the correlations of protein structure α-helix, β-sheet and α-helix to β-sheet ratio with nutrient profiles in

Table 3. Correlation between protein structure α-helix to β-sheet ratio and chemical profiles, Cornell net carbohydrate and protein system (CNCPS) protein fractions, *in situ* degradation kinetics and nutrient supply in the different types of co-products (wheat dried distillers grains with solubles (wheat DDGS), maize DDGS and blend DDGS with total sample number *n* 4) from bioethanol production (include just dependent variables that were significantly affected by the ratios)

	Protein secondary structure (α-helix, β-sheet and their ratio)					
	α-Helix		β-Sheet		Ratio of α-helix to β-sheet	
Items	Correlation coefficient (*R*)	*P*	Correlation coefficient (*R*)	*P*	Correlation coefficient (*R*)	*P*
Protein profiles						
ADIP (% of CP)	0·97	0·02	0·96	0·04	0·91	0·09
Protein subfractions						
PC (% of CP)*	0·97	0·02	0·96	0·04	0·91	0·09
Estimated intestinal dRUP						
dRUP (%)	−0·83	0·16	−0·80	0·19	−0·95	0·04

ADIP, acid-detergent insoluble protein; CP, crude protein; dRUP, digestibility of ruminally undegraded protein.
* PC = fraction of CP recovered with acid-detergent fibre, and is considered to be undegradable.

the DDGS with $P<0·10$. There was a tended correlation between the protein structure α-helix to β-sheet ratio and acid-detergent insoluble CP with R 0·91 ($P = 0·09$) (Table 3), but there were no correlations ($P>0·10$) between the protein structure α-helix to β-sheet ratio and non-protein N, soluble CP and neutral-detergent insoluble protein (data not shown). This result indicates that higher protein α-helix to β-sheet ratio is associated with a higher acid-detergent insoluble CP value in the bioethanol co-products.

During bioethanol processing, heat drying process is applied. Heat drying facilitates the Maillard reaction, through which sugar residues condense with protein amino acids, rendering proteins indigestible. These indigestible proteins are recovered in the lignin and acid-detergent fibre.

Table 4. Data obtained from regression analysis used to find the most important variables to predict protein nutrient supply using protein molecular structural parameters (α-helix to β-sheet ratio and amide I to amide II ratio) in the co-products (wheat dried distillers grains with solubles (wheat DDGS), maize DDGS and blend DDGS with total sample number *n* 4) from bioethanol production with tested regression model*

Predicted variables (*Y*)	Variable(s) selection (variables left in the model with $P < 0·05$)	Prediction equations (test model: $Y = a + b_1 \times x_1 + b_2 \times x_2$)	Model R^2 value	RSD	*P*
Protein values					
SCP (% of CP)	Ratio of amide I to amide II left in the model	SCP = −1·76 + 5·52 × R_I-II	0·98	0·38	0·01
PA (% of CP)†	Ratio of amide I to amide II left in the model	PA = −1·76 + 5·52 × R_I-II	0·98	0·38	0·01
PB2 (% of CP)‡	Ratio of amide I to amide II left in the model	PB2 = 120·18 − 29·54 × R_I-II	0·91	4·75	0·04
tdCP (% of DM)§	Ratio of amide I to amide II left in the model	tdCP = 15·05 + 7·74 × R_I-II	0·91	1·26	0·04
Rate and extent of protein rumen degradation *in situ*					
Kd (%/h)‖	No variables met the 0·05 significant level for entry in the model				
RUP (%)	Ratio of amide I to amide II left in the model	RUP = −171·94 − 83·43 × R_I-II	0·96	9·16	0·02
Estimated intestinal digestibility of RUP (dRUP)					
dRUP (%)	Ratio of α-Helix to β-Sheet left in the model	dRUP = 2·53 − 1·62 × R_α-β	0·92	0·02	0·04
Modelling nutrient supply using DVE system					
AMCP (g/kg DM)	No variables met the 0·05 significant level for entry in the model				
ENDP (g/kg DM)	No variables met the 0·05 significant level for entry in the model				
ARUP (g/kg DM)	No variables met the 0·05 significant level for entry in the model				
Total DVE (g/kg DM)¶	No variables met the 0·05 significant level for entry in the model				
OEB (g/kg DM)**	Ratio of amide I to amide II left in the model	OEB = 100·87 − 15·53 × R_I-II	0·93	2·20	0·04

RSD, residual standard deviation; SCP, soluble crude protein; CP, crude protein; tdCP, total digestible CP; RUP, ruminally undegraded protein; dRUP, digestibility of RUP; AMCP, absorbable microbial CP; ENDP, endogenous CP losses; ARUP, absorbable ruminally bypassed CP; NRC, National Research Council; CNCPS, Cornell net carbohydrate and protein system.
* Model: *Y* = ratio of α-helix to β-sheet (R_α-β) + ratio of amide I to amide II (R_I-II).
† Protein subfractions using the CNCPS include PA (fraction of CP that is instantaneously solubilised at time zero).
‡ Protein subfractions using the CNCPS include PB2 (fraction of CP that is intermediately degraded in the rumen).
§ tdCP (NRC-2001 summary approach).
‖ Kd = *in situ* degradation rate.
¶ DVE = total intestinally absorbed protein supply.
** OEB = degraded protein balance.

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Thus, an indication of the severity of the drying conditions can be provided by the content of acid-detergent insoluble CP. A negative relationship between acid-detergent insoluble CP and the ruminal and intestinal availability of DDGS protein was reported[28]. In the present study, a tended correlation between the α-helix to β-sheet ratio and acid-detergent insoluble CP was found, which may indicate that higher α-helix to β-sheet ratio may result in lower ruminal and intestinal protein availability of DDGS.

For Cornell net carbohydrate and protein system (CNCPS) protein fraction correlation, the results showed that the protein structure α-helix to β-sheet ratio had a positive correlation with the protein PC fraction ($P=0{\cdot}09$) with R 0·91, but that it had no correlation ($P>0{\cdot}10$) with protein PA, PB1, PB2 and PB3 fractions. The PC fraction was undegradable, and contained proteins associated with lignin and tannins and heat-damaged proteins. These results indicate that a higher α-helix to β-sheet ratio may result in a higher undegradable protein content in the DDGS.

For *in situ* parameter correlation, the results showed that the protein α-helix to β-sheet ratio had no correlation with soluble fraction, degradable fraction, undegradable fraction, Kd (the rate of degradation of the D fraction; /h) and RUP. The results also showed that there was a strongly negative correlation between the protein α-helix to β-sheet ratio and estimated protein intestinal digestibility of RUP *in vitro* (R − 0·95, $P=0{\cdot}04$). These results suggest that a high protein α-helix to β-sheet ratio may be detrimental to the protein availability of the DDGS in the intestine in terms of its use as a feed ingredient. This result is opposite to the previous findings that indicate that a higher β-sheet content results in lower nutrient availability. However, the heating methods were different (autoclaving *v.* dry heat) and this might be a part of the reason.

For predicted nutrient supply correlation, the results showed that the protein α-helix to β-sheet ratio had no correlation ($P>0{\cdot}10$) with absorbed microbial protein synthesis, endogenous CP losses, absorbable ruminally undegraded CP, total intestinally absorbed protein supply and degraded protein balance. However, in a previous study, the autoclaving of Vimy flaxseed changed the protein structure α-helix to β-sheet ratio, and decreased RUP and increased potential nutrient supply to dairy cattle. The protein structure α-helix to β-sheet ratio had a significantly positive correlation with total intestinally absorbed protein supply and a negative correlation with degraded protein balance[2]. These results suggest that different heating methods may have different impact on feed protein molecular structures.

Using protein structure profile as a predictor of the nutrient supply from bioethanol co-products

The results obtained from multiple regression analyses are shown in Table 4. The tested multiple regression model was: $Y =$ ratio of α-helix to β-sheet + ratio of amide I to amide II. This analysis was done to find a suitable protein structure variable that can be used to predict nutrient supply from the DDGS to dairy cattle.

The results showed that the ratio of α-helix to β-sheet was a better predictor of the estimated protein intestinal digestibility of the DDGS *in vitro* (with 92 % of the variance being accounted for). The ratio of amide I to amide II was a better predictor of the degraded protein balance of the DDGS (with 93 % of the variance being accounted for).

This is a novel approach to use protein molecular structure as a predictor of the protein nutrient availability in its preliminary stage. In order to obtain a more conclusive predictive equation, a large-scale *in vivo* study with various sources of bioethanol co-products is needed to test the applicability of the protein molecular structural parameters investigated. The development of a method to improve the estimation of protein value (protein degradability and digestibility) will highly benefit the scientific community.

Conclusions

In conclusion, the protein structure α-helix to β-sheet ratio in the DDGS had a strongly negative correlation with estimated intestinal digestibility of RUP, and it had no correlation with rumen degradation kinetics (rate and extent), total intestinally absorbed protein supply and degraded protein balance. The protein amide I to amide II ratio in the DDGS had a strongly positive correlation with the total digestible CP and strongly negative correlations with RUP and degraded protein balance, but it had no correlation with total intestinally absorbed protein supply.

The results indicate that the changes in the protein molecular structure α-helix to β-sheet ratio and the amide I to amide II ratio during bioethanol process, either due to enzymatic fermentation processing or due to final co-product drying, highly associated with estimated protein intestinal digestibility and degraded protein balance, can be used as predictor of the protein nutritive value in the DDGS sample from bioethanol processing. A large-scale *in vivo* study with various sources of bioethanol co-products is needed to test and verify the applicability of the protein molecular structural parameters investigated.

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