

Effect of Physical Processing of Pea (*Pisum sativum*) on Nitrogen Fractionation and Intestinal Protein Digestion

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Abstract

The aim of this study was to investigate the effect of different methods of physical processing of pea (*Pisum sativum*) on ruminal and intestinal digestion and protein fractionation using *in vitro* techniques. Raw pea unprocessed contain 95.8 dry matter, 22.9 crude protein, 35.4 NDF, 6.5 ADF, 6 crude fat and 1.2 minerals % per DM. Physical processing of raw peas was done using four methods 1: Soaking the peas in water, 2: Autoclaved pea for different times, 3: Microwaving of peas for 0, 2, 4, 6 and 8 minutes at 1000 watts and 4: Roasting peas for 30 minutes in a thermal tunnel. Among the various processing methods in this experiment, microwaved peas for 4 to 8 minutes and roasting caused to a reduction in NPN, rapidly degraded true protein (B1), and an increase in True protein with an intermediate degradation rate (B2) and true protein (TP). Comparing the effect of processing on digestibility of pea protein in the rumen and intestinal showed that roasting for 30 minutes at 100°C not only increased rumen degradable protein (RDP), but also increased the digestibility of protein in the small intestine.

Keywords: Protein digestibility; Pea seed; Feed processing; CNCPS

Introduction

Pea (*Pisum sativum*) appears to be the protein source best suited to the ecological and climatic conditions of many countries. The crop provides an opportunity for diversification and is probably the most suitable protein source for animals. Moreover, if it is to be used more efficiently in ruminant nutrition, in the diets of rapidly growing calves and high yielding dairy cows, the extent of protein degradation in the rumen must be reduced without altering its intestinal digestibility. Unlike cereal grains, peas are a good source of both energy and protein for animals. The CP fraction of peas, as with other leguminous seed, is highly soluble [1]. Pea protein is also characterized by high rumen degradability.

To evaluate and include any feed in ruminant diets is the most important way to meet livestock requirement. One of these models is Cornell Net Carbohydrate and Protein System (CNCPS) [2,3] which is increasingly used to regulate diets of dairy cows. In the CNCPS model, the protein is divided into three general components of A, B and C [3]. Nonprotein nitrogen is denoted as the A fraction while true protein is broken down into B1, B2 and B3 fractions based on decreasing solubility. The respective fractions are dependent upon the estimation of insoluble nitrogen, true protein, and the nitrogen residual in ADF and NDF. The nitrogen that is insoluble in acid detergent is denoted as the C fraction and is assumed to be indigestible. Fraction C is ADIP, which is unavailable and unusable for livestock due to the connection to acid detergent insoluble fiber [3-5]. The CNCPS has been used as a farm management tool to optimize use of "home grown" feeds, decrease the need for purchased supplements, optimize herd size and improve the annual return over feed cost [6]. The CNCPS was first published in 1992 and 1993 in a series of four papers [3,7-9], but the model has been continually refined and improved over the last 10 years [10-16]. Current recommendations for feeding proteins to cattle are

based on the concept of absorbable protein [17]. The total amount of protein available for absorption is dependent on the flow of microbial and dietary N to the duodenum and their respective intestinal digestibilities. Variation in *in vivo* intestinal digestion among protein supplements has been reported [18,19]. Estimates of protein digestion in the small intestine is expensive and labor-intensive, and it requires the use of surgically prepared animals. Various *in vitro* methods have been developed, including ADIN (enzymatic procedures [20] and an *in situ* mobile-bag technique [21]. Development of an enzymatic *in situ/in vitro* technique to estimate intestinal digestion of proteins may provide the means to determine intestinally absorbable dietary protein of individual feeds [22]. This technique is 1) simulate physiological conditions of ruminants, including potential effects of ruminal fermentation; 2) rapid, reliable, and inexpensive; 3) applicable to a wide variety of protein supplements, and 4) accurately reflect differences in protein digestion. Literature on the nutritive value of processed peas for ruminants is scarce [1]. The objectives of this study was to determine nitrogen fractionation of various processed peas based on CNCPS model and ruminal and intestinal degradability of pea proteins using a three-step enzymatic *in situ/in vitro* technique.

Materials and Methods

Chemical analysis

The pea samples were dried in a forced-air oven (65°C) for 48 hours, ground to pass a 2 mm screen and analysed for the total N (Kjeldahl method, Kjeltec 2300 Auto analyzer, Foss Tecator, Sweden), neutral detergent and acid detergent fiber ([NDF and ADF], [23]), ether extract and ash [24]).

Physical processing

Processing of raw peas was done using four methods including 1: soaking the pea samples in water for 24 hours at 37°C in water bath, 2:

autoclaved samples at 30, 60 and 90 minutes, 3: microwaved for 0, 2, 4, 6 and 8 minutes at 1000 watts and 4: Roasted for 30 minutes at 100°C in a thermal tunnel.

Nitrogen fractionations (CNCPS model)

The chemical methods used to determine the nitrogenous sections of peas were based on the recommendations of Licitra et al. [25].

NPN (Non Protein Nitrogen): First weigh 0.5 g dry ground sample, then add 50 ml of cold distilled water. After add 8 ml of 10% sodium tungstate solution. Let glass bottle stand at 20-25°C for 30 min. Bring pH to 2 by adding 10 ml of 0.5 M sulfuric acid and let flask stand overnight at room temperature. Filter with mild vacuum. Wash residue and determine residual nitrogen. Calculate NPN by subtracting residual nitrogen from total nitrogen. Value of NPN may be expressed as crude protein ($N \times 6.25$) or as percent of total sample nitrogen.

BSP (Buffer Soluble Protein): About 50 ml of borate-phosphate buffer to 0.5 g of sample in a glass bottle was added. Next, adding 1 ml of sodium azide 10%, the resulting solution was shaken for 3 hours at normal laboratory. Then, the solution was filtered and the residual nitrogen was determined using Kjeldahl method. The total crude soluble protein content was calculated by subtracting nitrogen of residual from total pea nitrogen and it's multiplied by 6.25. However, the true soluble protein content was calculated by subtracting residual nitrogen from insoluble nitrogen in the NPN determination method.

NDIN (Neutral Detergent Insoluble Nitrogen): Transfer 0.5 g of the sample into Dacron bag and place them in a NDS (60 ml per bag), then place it in an autoclave at 100°C for 75 minutes. Then rinse the bags and use the residual to measure the nitrogen using Kjeldahl method.

ADIN (Acid Detergent Insoluble Nitrogen): The method of determining the ADIN was similar to the NDIN method, but instead of the NDS solution, the ADS solution was used.

In Situ Ruminant and Post-Ruminal Disappearance

Ruminal protein degradability

Two ruminally fistulated Holstein steers were used which received twice daily a diet consisting of forages and concentrate (50:50, on a dry matter basis). Fresh water was available at all times. Samples of processed and unprocessed peas were milled through a 1 mm screen, then weighed into 10 cm × 16 cm nylon bags with pore size of 42 µm. Six bags were attached along 25 or 13 cm of flexible polyvinylchloride tubing and secured to the ruminal cannula plug by means of a 70 cm nylon line. The bags were incubated in the ventral sac of the rumen for three consecutive periods in triplicate for each incubation time in each period and in each steer. They were removed from the rumen at 12 h. Bags were then briefly rinsed under cold tap water and machine washed three times for 5 min each with fresh water and finally dried at 60°C for 48 h in a forced air oven, then weighted and N of residual determined [26].

Intestinal digestibility of ruminal undegraded protein

An *in vitro* pepsin digestion assay adapted from AOAC [27] was used to determine the effect of HCl-pepsin predigestion on protein digestion by pancreatin. Residues containing 15 mg of N were preincubated with 10 mL of 0.1 N HCl solution containing 1 g/L of pepsin (Sigma P-7012, Sigma) at pH 1.9. Samples were incubated for 1

h at 38°C. After incubation, pH was neutralized with 0.5 mL of a 1 N NaOH and 13.5 mL of a buffer pancreatin solution (0.5 M phosphate solution, pH 7.8, containing 3 g/L of pancreatin [Sigma P-7545, Sigma]) were added. For the zero time, the HCl-pepsin solution was immediately neutralized. Samples were vortexed and incubated at 38°C for 24 h in a shaking water bath. After incubation, 3 mL of a 100% (wt/vol) TCA solution were added, samples centrifuged at 10,000 x g for 15 min, and TCA-insoluble N measured. After all, the N of 5 cc of residual determined [26].

Statistical analysis

The data of the 3-step enzymatic procedure were calculated as described by Calsamiglia and Stern [26]. Data were analysed by SAS [28] using the general linear model procedure as a completely randomized design. The statistical differences were determined using Duncan's multiple range tests at $P < 0.05$.

Results and Discussion

Pea (*Pisum sativum*) used in the present study had (% per DM) containing 95.8 DM, 22.9 CP, 35.4 NDF, 6.5 ADF and 6 EE. Comparison of nitrogen fractionations of pea (g/kg CP) with various processing method based CNCPS model are shown in Table 1. In the present study, a significant ($P < 0.05$) difference was found between non-protein nitrogen (NPN) fraction of pea, which are commonly rapidly degrade fraction in the rumen; Processed pea at autoclaves at various temperatures, Ben-Marie at various temperatures and spitting at 30 minutes compared with processed and unprocessed peas significantly decreased. In the study of Fathi Nasri [29] and Ganesh and Grieve [30], it has been shown that heat treatment of protein sources (soybean) reduced the percentage of NPN. In fact, as regards the most part of the NPN is free amino acids and peptides, heat may be due to deformation and their placement in the sediment [31]. The results of this study showed that heat reduced the NPN of peas. Heat treatment with autoclave at different temperatures had no effect on B1 fraction of pea compared with the control group and Ben-Marie, but under the rays of peas in the microwave for 2 to 8 minutes after soaking for 24 hours at 37°C in Ben-Marie reduced the amount of buffer soluble protein (B1). Roasting (spit out) of peas in the tunnel temperature of 100°C, the amount of B1 sharply reduced compared to unprocessed peas. Findings of previous researchers [29,32,33] also indicated that the heat processing of protein sources reduced B1 fraction (BSP). In fact, it has been found that commonly soluble proteins in protein sources are usually albumins and globulins, which are rapidly degraded by rumen microorganisms [3]. These proteins are highly sensitive to heat and cause area of the protein degrading microbial enzymes to be deactivated and generally reduce the amount of soluble proteins in the protein sources, which is similar to the results of the present study [34]. The study of Mustafa et al. [35] showed that the heat of peas in autoclave at 127°C for 10, 20 and 30 minutes did not affect crude protein but did the non-protein nitrogen (NPN) and true soluble protein reduced compared to the control group. As regards the content B1 true proteins was reduced from 537 g/kg of crude protein to 49, 31 and 18 g/kg in 10, 20 and 30 minutes of autoclave, respectively. Crude protein solubility of feed protein sources have been well documented as a result of heat treatment [36]. The results of this study showed that autoclaving of peas at different temperatures did not affect the B2 fraction of pea, but under the rays of peas in the microwave for 6 and 8 minutes after soaking the peas for 24 hours at 37°C in Ben-Marie increased the B2 fraction (NDSP) compared with unprocessed

peas. The study also showed that roasting of peas at 100°C increases the B2 fraction compared with unprocessed peas, as the amount of B2 fraction increased from 9 g/kgCP to 15 g/kgCP. Results of previous studies have shown that heat processing has increased the B2 fraction in protein sources such as soybean meal and cottonseed meal [33,37]. This fraction of protein usually contains true proteins and large peptides, some of which are digested in the rumen and the rest are transmitted to the intestine. The fate of this fraction depends on the digestion rate and passage rate of feed. Therefore, heat processing of protein sources reduces B1 and increases B2 fractions. Decreasing B1 and increasing B2 fractions are associated with increasing rumen undegradable protein (RUP) and decreasing rumen degradable protein (RDP), therefore improving the efficiency of feed protein sources [3,38]. The acid detergent soluble protein (B3) were similar in peas between different treatment groups and control group. Study of Ganesh and Grieve [30] showed that roasting raw soybean at 125 and 150°C reduced B3 fraction, but increased with temperatures up to 165°C, that indicating heat damage. The results of our study showed

that acid detergent insoluble protein (C) were similar in peas between different treatment groups and control group. Therefore, the heat treatments in this experiment are not severe enough to make existing proteins inaccessible through the Millard reaction. A study by Mustafa et al. in [35] showed that the heat of raw peas in autoclave at 127°C for 10,20 and 30 minutes increased B3 with increasing temperature in processed peas, but there was no effect on the C fraction of the protein. In relation to B3 fraction, it increased from 23 g/kgCP in the raw pea (control) group to 60, 77 and 123 g/kgCP in 10, 20 and 30 minutes of autoclave. In general, the results of protein fractionation showed that firstly processing by different methods can cause changes in various protein fractions and possibly a change in the rate of degradability of pea proteins. Secondly, among different methods of processing in this experiment, microwaves of peas for 4 to 8 minutes, as well as roasting peas at 100°C for 30 minutes due to decreasing NPN, B1 and increasing B2 and TP and no effect on B3 and C fraction, which in total indicates less rumen degradation and no change in the digestibility of peas proteins.

	CP ¹	NPN(A)	TP	BSP(B ₁)	BIP	NDSP(B ₂)	NDIP	ADSP(B ₃)	ADIP(C)
Raw peas (Control)	28.21	8.46	19.76	4.65	15.11	9.74	5.36	2.34	3.02
Ben marie 24 h	27.16	8.03	19.13	5.31	13.82*	8.74	5.07	2.07	3
Autoclaved (30 min)	26.57	5.25*	21.32*	6.02	15.3	9.97	5.32	2.89	2.44
Autoclaved (60 min)	28	6.59*	21.40*	6.88	14.52	9.08	5.44	2.23	3.21
Autoclaved (90 min)	26.72	4.82*	21.90*	6.72	15.19	9.51	5.68	2.87	2.81
Microwaved (2 min)	28.17	9.51	18.66	2.85*	15.81*	9.16	6.65	3.92	2.73
Microwaved (4 min)	28.59	6.99*	21.61*	2.13*	19.47*	14.25*	5.22	3.03	2.19
Microwaved (6 min)	28.03	6.22*	21.81*	2.32*	19.50*	14.32*	5.18	2.67	2.5
Microwaved (8 min)	27.3	6.07*	21.23*	0.59*	20.64*	15.31*	5.33	2.73	2.61
Roasted (30 min)	28.5	6.19*	22.30*	0.79*	21.52*	15.01*	5.5	2.83	2.67
SEM	0.47	0.58	0.58	0.46	0.33	0.51	0.64	0.53	0.21
P-value	0.0529	0.0003	0.0019	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0974

Table 1: Effects of physical processing on protein analysis and nitrogen fractionation of peas (g/kg CP) based on CNCPS model. 1 CP=Crude protein, NPN (A)=non protein nitrogen, TP=true protein, BSP (B₁)=buffer soluble protein, BIP=buffer insoluble protein, NDSP (B₂)=neutral detergent soluble protein, NDIP=neutral detergent insoluble protein, ADSP (B₃)=acid detergent soluble protein, ADIP (C)=acid detergent insoluble protein. *Means within a column with star were significant differed from the control (P>0.05).

Ruminal and intestinal disappearance of protein of raw and processed peas are shown in Table 2. The results of this experiment indicated that some pea processing methods have a significant effect on the percent of protein disappearance in rumen and intestine. The percent of ruminal protein disappearance in raw peas in our experiment was about 90% and autoclave treatment at different temperatures did not significantly change the percentage of crude protein disappearance in the rumen. But, it was shown that microwave processing of peas for 2 to 8 minutes and roasting at 100°C for 30

minutes significantly reduced the crude protein degradation in the rumen. Previous studies have shown that heat processing reduces the ruminal degradation of dry matter and crude protein in processed protein sources compared to the raw pea. According to a study by Guelema et al. [39], the pea extruded at 140°C reduced the ruminal protein degradation from 88% to 66%. However, steam flake of pea only reduced the rumen's nitrogen from 69 to 62 percent, which is very slight and insignificant compared to the change in the flow of amino acids in the cereals due to flaking. Therefore, it can be said that steam

flake has no significant effect on ruminal protein degradability of pea [1]. In fact, it has been shown that heat by changing the structure of proteins and creating bridges across within peptide chains and between peptide chains with carbohydrates cause decreases the solubility of protein, reduces protein availability for microbial enzymes and reduces rumen degradation rate. According to studies of Guelema et al. [39] and Petit et al. [40] the high digestibility of pea protein in the rumen can be a limiting factor for peas when it can be a good alternative to ration protein sources, especially when there is a need for RUP in high-producing dairy cows.

There are several ways to increase RUP in the diet, the most common of which is thermal processing [39]. Heating creates carbonyl groups of sugars that combine with free amino acids in the reaction of Millard. In a study by Mustafa et al. [35] it was shown that autoclaved peas for 10 to 30 minutes had a less rapid degradable protein and had a more middle degradable protein than the raw peas. The heat treatment method, which could improve digestibility for leguminous such as peas, was toasting with pressure. After processing, it was found that ruminal protein degradation of pea was reduced by 29%, although the total protein digestibility was still high. The results of this experiment in general showed that none of the pea processing methods had significant effect on the degree of degradability of the part of the protein that passed through the rumen and reached the small intestine and only roasting of pea significantly increased the degradability of the transmitted protein in the small intestine (Table 2). The high nitrogen pepsin solubility (95.8%) of peas extruded at 140°C demonstrate that extrusion had little or even no negative effect on intestinal digestibility [41]. The rate of passing protein digestion in the small intestine varied between 65% and 83% in raw pea and roasted pea, respectively. However, the results of protein digestibility for microwave samples were almost similar to that of roasting method, but did not have a statistically significant difference with the raw pea. As noted above, previous studies have shown that heat processing reduces the degradation of dry matter and protein in feed protein sources compared to their raw sample. Looks temperature with changes in protein structure prevents access the rumen microorganisms and increased to pass it. But the usefulness of this method depends on the determination of digestibility of the passing protein to the small intestine. If post ruminal protein digestibility is not reduced, the nutritional value of the protein increases. The results of Mustafa et al. [35] showed that the processing of peas at 120°C in the autoclave increased the passing protein but did not affect the protein digestibility of the experimental samples. In present study, the autoclave of peas did not change the percent of protein degradation in the rumen and non-significant increase protein digestibility in the small intestine (Table 2). The microwaving method in different times reduced the protein degradability of raw peas and non-significant digestion in the intestine. The roasting method for 30 minutes at 100°C not only increased the protein degradation in the rumen, but also increased digestibility of protein in the small intestine.

	CP disappearance (%)	
	Ruminal	Intestinal disappearance of ruminal indigestible fraction
Raw peas (Control)	90.92	65.62
Ben marie 24 h	88.1	64.65
Autoclaved (30 min)	92.83	62.71

Autoclaved (60 min)	93.13	73.39
Autoclaved (90 min)	91.73	71.93
Microwaved (2 min)	86.17*	64.65
Microwaved (4 min)	85.42*	79.21
Microwaved (6 min)	87.07	80.18
Microwaved (8 min)	79.86*	75.33
Roasted (30 min)	84.38*	83.98*
SEM	2.35	4.93
P-value	0.008	0.0484

Table 2: *In situ* ruminal and post-ruminal disappearance of crude protein (g/kg of CP) of peas. *Means within a column with star were significant differed from the control (P>0.05).

Conclusion

Results of chemical composition of the pea samples indicated that it is a feed source rich in protein, however based on nitrogen fractions analysed in the present study the protein is highly degradable in the rumen. The processing of raw peas with different methods of physical treatments in total showed that, firstly, processing by different methods could cause different changes in different protein fractions and possibly a change in the rate of rumen degradability of pea proteins. Secondly, among different methods of processing in this experiment, the microwave of raw peas for 4 to 8 minutes and the roasting at 100°C for 30 minutes caused a decrease the rapid degradation fraction in rumen and increased slow release fraction in rumen that in total, it shows that more protein passes through the rumen, they are the best pea processing methods. Following the confirmation of this issue and investigating the digestibility of passing protein from the rumen in the small intestine, the results of determination of protein digestibility by a three-step enzyme method showed that among the processing methods, only roasting method not only increased the amount of protein passing, but also It increased protein digestibility in the small intestine. After comparing the effect of different processing methods on raw peas using CNCPS method and three-step enzymatic method, it was determined that the roasting of peas for 30 minutes at 100°C would be the best treatment method in order to increase the passage of raw pea protein and increase digestibility in the small intestine than other treatments.

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