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Title: The effect of red clover (*Trifolium pratense* L.) polyphenol oxidase on protein complexing and utilisation in forage-fed ruminants

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The effect of red clover (*Trifolium pratense* L.) polyphenol oxidase on protein complexing and utilisation in forage-fed ruminants

Gareth O'Keeffe[1], Dr Alison Kingston-Smith[2] and Prof. Nigel Scollan[2]

[1]15 Provident Cottages, Maesycwmmer, Caerphilly County Borough, CF82 7SA
[2]IBERS, Aberystwyth University

Supervisor: Dr Alison Kingston-Smith

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1. Abstract

Protein utilisation in forage fed ruminants is notoriously inefficient with much of the ingested protein not being used in milk and meat production because of rapid post-ingestive protein breakdown. This work examined the potential to exploit the properties of the enzyme polyphenol oxidase (PPO) to improve fresh forage feeding. PPO catalyses the conversion of phenols to quinones, which can then bind to protein resulting in production of large complexes which are thought to be resistant to proteolysis. PPO has been shown to improve protein preservation in silage but its role in grazing has not been explored.

The effect of PPO on protein protection was investigated in the forages grasses cocksfoot, Timothy and perennial ryegrass, which had varying PPO activities, plus wild type red clover and a low-PPO mutant. Forage offtake from field plots and newly ingested feed down boli were analysed for PPO activity, dry matter (DM), nitrogen (N) and protein quantity and quality. Total PPO activity varied significantly between species but was found to be approximately 50 % activated in the field regardless of forage type. In all cases significant amounts of PPO activity was lost as a result of harvesting and transport conditions. Greatest losses between field to farm were observed in red clover wild type with much of any remaining activity lost in down-boli. Cocksfoot contained the highest PPO activity of the grasses studied but lost ~90% of activity between field to farm, although activity was retained in the down boli. As there was no evidence for conversion of active to latent form of the enzyme it is proposed that the PPO protein was damaged irreversibly during transportation. Loss of PPO activity meant that predicted activity differences were not realised at point of animal feeding, which would have impacted on bolus quality and potential effectiveness of the PPO trait. Accordingly, regardless of species, bolus generation resulted in minimal changes in %DM, %N or extractable protein with no evidence for enhanced formation of protein complexes in species with higher endogenous PPO activity (red clover and cocksfoot) as opposed to low PPO containing species.

In vitro proteomic analyses were performed to identify target proteins for PPO-mediated protection of protein from proteolysis. Red clover protein extracts had distinct proteome profiles depending on whether PPO was active or inhibited by inclusion of ascorbate. Regardless of treatment there was no evidence for production of high molecular weight protein complexes, although PPO activity was associated with enhanced abundance of RuBisCo breakdown products after tryptic digestion. Two dimensional gel electrophoresis revealed 20 polypeptide spots unique to the presence of PPO activity. Peptide sequencing by tandem mass spectrometry showed these to originate from four membrane-localised, chloroplastic proteins. There was no evidence that PPO affected protease proteins.

In conclusion, exploiting PPO to promote increased protein use efficiency in ruminants will involve a combination of management and genetics. Observed losses of PPO in transport experiments have implications for zero-grazing feed strategies. Although formation of protein complexes was not a significant factor in these studies, low level PPO-mediated protein protection through preservation of breakdown products may be effective in animals. It appeared that PPO activity affected specific chloroplastic protein targets in red clover, although the reason for this is unclear at present. It is concluded that future efforts should address increasing the endogenous activity of PPO, particularly in preserving the proportion of the enzyme in the active rather than latent state. Consideration should also be given to identifying targets for PPO-mediated protection from proteolysis so that they can be increased in forage breeding programmes.

2. Introduction

Objectives: Legumes contain a high protein content which is a desirable component of ruminant feed. However, because of the inherent inefficiency of protein transformation in the rumen and the involvement of plant and microbial proteases, it is proposed that in the presence of sufficient PPO activity protein-quinone complexing will occur which will have the effect of decreasing the extent of proteolysis in the newly ingested fresh forage down bolus. This will allow a greater proportion of the forage protein to escape the rumen and be digested in the abomasum, where (plant and microbial) protein breakdown products absorbed into the animal's bloodstream are used to drive animal growth.

Record of Research: Red clover wildtype 'Milvus' with high recordable levels of PPO activity and a low-PPO mutant variety, along with grasses of differing PPO activity; cocksfoot, perennial ryegrass and Timothy grass, were chosen for comparative activity of PPO or lack thereof. Both *in vivo* and *in vitro* experimentation was designed to generate boli in comparable conditions for analysis by various means. Red clover and grass species experiments were separate, but utilised the same experimental animals. An early experiment was designed to assess the physical damage inflicted on plant tissues, utilising evans-blue vital stain and photography. Protein was extracted using standard methodology, modified where appropriate, either using HEPES or LE-LiDS (Mae *et al.* 1993) extraction buffers, modifications included the addition of PVP and/or the addition of ascorbate was included to prevent the formation of complexed protein (Walter and Purcell, 1980; Winters and Minchin, 2001; Webb *et al.* 2014). Protein was quantified using spectrometry; Bradford protein assay or micro-Lowry micro assay (Lowry et al. 1951; Winters and Minchin, 2005), using BSA as a standard. PPO was extracted, isolated and had activity measured using existing methodology (Robert *et al.* 1995; Winters and Minchin, 2001; Winters and Minchin, 2008). Units of PPO activity (referred to as units /g DW) where original absorbance of 30 sec was converted to absorbance over one minute ($AU._{420}(60/30)$)=A, and converted to DM weight of sub sample (A*(1/0.05g))=B, and then converted to $AU._{420}$/sec (B/60), giving $1\Delta AU._{420}$/sec. Proteins were analysed using SDS Page (either hand cast or precast) where proteins were separated in one plane (size), and stained for visual analysis with Coomassie blue, or by silver staining (Kerenyi and Gallyas, 1973). Analysis of the abundance of specific polypeptides (Rubisco) was achieved utilising Western blotting, transfer to nitrocellulose, and blots used for immunodetection of RuBisCo (LSU and SSU), bands of cross-reaction were detected by incubation with horseradish peroxidase. Images of SDS page gels and Western Blotts were taken by densitometer. Further analysis of proteins was possible using 2D proteomic techniques, where protein sample is separated on 2 planes (size and charge). This process was not applicable in this instance to bolus material due to humic interference. Protein prepared as for 1D analysis, and further purified to increase clarity (Morphew *et al.* 2007). Proteins were transferred to rehydradion strips, and then loaded onto hand cast 2D gels, and completed gels were stained with Coomassie brilliant blue. Gels were imaged by

GS-800 densitometer, and analysed using Progenesis PG220 v.2006 utilising non-spot background subtraction on average gels, created from >3 biological replicates. Unmatched spots were excised and sent for further identification (Dr Ian Brewis, Cardiff University) and analysis against a red clover protein map, and by MASCOT. Further analysis of identified proteins was also conducted using online databases; initial basic local alignment search tool (BLAST), the UNIprot database (BLAST AND FASTA) and for further identification using SignalP (cleavage sites), PROSITE functional analysis and KEGG metabolic pathways. Statistical methods were conducted using Genstat 13th Edition, with separate analysis described for each experiment.

Why the work was necessary: the possibility to improve animal efficiency, through a naturally occurring plant enzyme, in crops already utilised by livestock farmers was worth investigating. Live research animals (under Home Office license) were employed to produce both realistic damage effect to plant tissues, and to allow the investigation of effects on PPO, potential complexing within the mouth in a small time frame, and as naturally formed down boli. Animals were utilised for the minimum time possible, and in the minimum number of experiments possible for results. The identification of PPO in the selected species, as well as recorded activities, were already in existence, and some knowledge existed in terms of silage production. However, very little knowledge existed as to the effects of PPO on plant protein both *in vivo* and *in vitro*. This research has provided new insight into the potential difficulties involved in observing PPO enzyme's activity during the production of down boli, and into proteins that are naturally resistant to tryptic digest in rumen like conditions.

3. Materials and methods

3.1. Chemicals and laboratory supplies

All chemicals used were of analytical reagent grade and obtained from Sigma -Aldrich Company, Ltd (Dorset, UK) or Fisher Scientific (Leicestershire, UK) unless otherwise indicated. Liquid nitrogen was supplied by Cryoservice (Worcester, UK). All plasticware and glassware, including pipette tips and microcentrifuge tubes were sterile and were purchased from Calbiochem, Merck Chemicals Ltd., Nottingham, UK, Fisher Scientific, Leicestershire, UK and Starlab, Milton Keynes, UK.

3.2. Plant material

3.2.1. Red clover

Red clover (Aa $_{43}$8$_{1}$ *Trifolium pratense* L. cv. $_{1753}$ 'Milvus') and the low PPO mutant (ABY-Aa 45^{21}) (Winters *et al.* 2008; Lee *et al.* 2004) were grown from seed in 30 cm pots of Levingtons Multipurpose compost, watered regularly and maintained under glasshouse conditions with supplemental lighting to compensate for day light reduction (as needed). Plants were established in 2009 and maintained in the vegetative state by regular cutting to 5 cm above base to encourage new growth. Plant material was cut with scissors for experimental use after at least 2 weeks regrowth. For experimental use plant material was either harvested *in situ*, weighed and then immediately frozen in liquid nitrogen or entire plants still in the pots were temporarily removed from the glasshouse for immediate use in the laboratory.

Alternatively, red clover (cv. Milvus; 0.7 ha) was sown at a rate of 6.5 kg /ha on 7th May 2009; Red clover (AA4512; 0.7 ha) 6.5 kg /ha on 7th May 2009 at Cae Gwastad, Gogerddan, Aberystwyth. Fertiliser P_2O_5 89 kg /ha and K_2O 89 kg /ha were applied to both clover plots on 6th May 2009, and then at yearly intervals with applied anti grass and/or broad leaf herbicides.

Both varieties of red clover were also sown at Cae Gwastad received Spring fertilisation (0.24.24 fert, P_20_5 88 kg /ha and K_20 88 kg /ha). Plants were also sown on 03 August 2011 for use in spring 2012, in pre-ploughed and fertilised land at Cae Penlon (Trawscoed). Plots of 2 m by 10 m with 0.1 m gaps separating each plot were devised, pegged and strung out to ensure no cross sowing. Viable seed of each species was mixed with a large quantity of dead rye-grass seed, and sown via broadcast in three repeated layers. After sowing, soil was raked to 2 cm over the seed, until no seed was visible. The sown area was then rolled to compress the soil allowing for good growth and preventing seeds being easily dug up by animals. Red clover forage was also cut via Holdrup and transported in a van under ambient temperature conditions and offered to animals in the same manner.

3.2.2. Grasses

Grass forage was obtained from SERF field plots established at Gogerddan Campus, Aberystwyth University, in 2008. Plots of 5x5 m^2 were established in 2008 and were designed as

mono cultures with guard plots to prevent cross contamination of plant species. These were replicated plots, which allowed cutting at 3 periods during the year, each cutting involved cutting five replicated sward blocks at weekly intervals. The field plots (5 m x 1 m) were situated ~8.2 miles /30minutes and were of 6 grass varieties comprising AberTop, AberDovey, AberEpic, AberDart, S.48 and Aber Magic in randomised order, over 4 repeated plots, segregated by AberDart guard plots. Sown in 2007 on soil of the Rheidol series; AberDart sown at 12.6 kg/ha, Abertop sown at 20 kg/ha, S.48 sown at 18 kg/ha. All plots harvested three times in each of the following three harvest years by Holdrup 1500 plot harvester (J. Haldrup a/s, Løgstør, Denmark at a cutting height of 5 cm. Plots received a total of 90 kg N ha^{-1} yr^{-1} applied n three equal doses.

For experimentation AberTop (cocksfoot *Dactylis glomerata* cv. Abertop), AberDart (perennial ryegrass *Lolium perenne* cv. Aberdart) and S48 (Timothy grass *Phleum pratense* cv. S.48) were selected.

Grass species (Aberdart, S.48 and Abertop) were sown at Cae Penlon (Trawscoed) in plots of 2m by 10m with 0.1m gaps on 01 August 2011 and viable seed of each species (AberDart sown at 12.6 kg /ha, Abertop sown at 20 kg /ha, S.48 sown at 18 kg /ha) was sewn via broad cast with a large quantity of dead ryegrass seed in three repeated layers. After sowing, soil was raked to 2 cm over the seed, until no seed was visible. The sown area was then rolled to compress the soil allowing for good growth and preventing seeds being easily dug up by animals. Grass forage was designated to be cut via Holdrup and offered to animals (but was not utilised).

3.2.3. Harvesting and transport

For experimental use relevant fields and plots were harvested in the morning (between 0900-1000) to minimise the time experimental animals were withheld food and as the plots from which material was cut was (in the case of red clover) designated for silage production and required wilting time, while grasses gathered from SERF plots was designated for drying over day and night and so had to be cut as early as possible. Red clover was harvested at a height of approximately 5 cm via Holdrup 1500 plot harvester (J. Haldrup a/s, Løgstør, Denmark) from Cae Gwastad on 15th July 2010 and 20th May 2011, and grasses were harvested from Gogerddan on 25th August 2010. Plant material was transported from Cae Gwastad in plastic sacking forage bags

in an enclosed vehicle to Trawscoed farm (8.2 miles East of Plas Gogerddan fig 2.1.). Sub samples of red clover and grasses were routinely removed (at harvest and at feeding) for protein quantification and fresh (FW) /dry weight (DW) analysis and proximate analysis (nitrogen and fibre) as indicated.

Grass forage (Aberdart, S.48 and Abertop) was harvested via Haldrup 1500 plot harvester (J. Haldrup a/s, Løgstør, Denmark) and a subsample of each forage was taken at the field site and flash frozen in liquid nitrogen, material was then transported in animal feed bags by van at ambient temperatures and a subsample taken on site at Trawscoed. Feed was then stowed in animal feed troughs until offered at consecutive intervals in a randomised order, based on the experimental design.

3.3. Use of animals

The work involved use of 4 non-lactating Holstein × Friesian cows, equipped with a 100 mm diameter rumen cannula. Prior to experimentation the cows were offered hay and red clover silage (*ad lib*) and allowed to graze freely for two weeks before experiments in mixed grass pasture. To encourage feed uptake for adequate bolus production the cows were fasted overnight immediately prior to the experimentation, which commenced at 09:00 h. All animal procedures and the care for the animals were carried out under strict regulations described in the Animals (Scientific Procedures) Act 1986 issued by the Home Office of Her Majesty's Britannic Government.

3.3.1. Bolus collection

Four non-lactating Holstein-Friesian cows were held in adjacent stalls in a randomised order for each experiment. Rumens were emptied completely one half hour before experimentation took place and contents stored. Animals were presented with red clover or grass in pairs, based on a randomised block design. Animals were allowed to feed until a maximum seven down boli had been generated. Individual boli were caught by licensed individuals through rumen cannula at the oesophageal junction. Each individual bolus was placed in a clean collection bucket, and then passed to washing stations. Individual boli were washed accordingly using hand dip, rinse and

squeeze in clean tap water (repeated), before being placed in muslin. Boli were clearly labelled and stored according to the individual method.

Each session of production of seven boli took approximately five to ten minutes, and animals were given a maximum of fifteen minutes to accept or refuse before feed was removed from their stall. Once all animals had been given an opportunity to feed, according to the method, rumen contents were replaced and cannula resealed. The health and wellbeing of each animal was of high priority, therefore time spent with empty rumens and in generating boli, was kept to a minimum in accordance with the terms of the licence (1 hr maximum – rumens were emptied as cropping and transportation of forage began).

Experiment 1 (TR211): Four overnight fasted Holstein × Friesian cows (Identification #1403, #3055, #5391, #6343) were offered one of three forages; cocksfoot (*Phleum pratense* cv AberTop), Timothy (*Phleum pratense* cv S48) or perennial ryegrass (*Lolium perenne* cv AberDart) as fresh forage cropped via Holdrup from Gogerddan Campus, Aberystwyth University in 2008 and transported to animals held in stalls. Animals were offered forage on an ad libitum basis, or until a sufficient number of down boli were formed and captured. Failures to feed were recognised after ten to fifteen minutes of withholding from forage. The experimental design was a four by four Latin square consisting of four periods with three treatments per period (one repeated). Each individual bolus was processed by rinsing in distilled water before it was weighed and divided into aliquots which were either stored at -20°C until further analysis or freeze dried for storage. Statistical analysis of forage quality was undertaken using ANOVA, with removal of cow*cow variation (Genstat 13.3 SP2 2011).

Experiment 2 (TR211B): In a slight modification of exp.1 experimental design, forages were offered as a three by four by Latin square, consisting of three periods with three treatments but the repeated treatment was a different selected forage (1 Perennial Ryegrass, 2 Cocksfoot, 3 Timothy) per period, and quantification, assay and statistical methods as exp.1, an example of the experimental design is below.

Period	Animal			
	#1403	#3055	#5391	#6343
1	3	1	2	2
2	2	3	1	1
3	1	2	3	3

Experiment 3 (TR217): Four overnight fasted Holstein × Friesian cows (Identification #1403, #3055, #5391, #6343) were offered one of two forages; red clover (*Trifolium pratense* cv Milvus) or a low-PPO mutant as fresh forage cropped via Holdrup 1500 plot harvester (J. Haldrup a/s, Løgstør, Denmark) from Cae Gwastad Gogerddan Campus, Aberystwyth University in 2011 and transported to animals. Animals were offered forage in the same manner as described in exp.1. In an experimental design taken from Kershner & Federer (1981) a four by four Latin square consisting of four periods with two repeated treatments per period animals were given consecutive offerings in an alternate order. Quantification, assay and statistical methods were as described for exp.1.

3.3.2. Bolus preparation and analysis

Collected down boli were initially rinsed by hand under running water within a 1mm sieve until saliva and fluid had been removed. However, this method was later modified to reduce possible losses in bolus material. Collected boli were placed directly into pre-cut muslin squares, which were sealed with plastic label and elastic. The boli were washed individually for 45 seconds in bucket of cold water (immersed five times, squeeze while immersed, immersed five times, squeeze out water) before being immediately frozen at -20°C.

Prepared boli were weighed before being freeze dried at -40°C for at least 3 days (or until fully desiccated), before being re-weighed and ground to powder. Freeze-dried ground boli were stored in individual pre-labelled containers.

Alternatively, boli were frozen at -20°C and divided into quarters while still frozen by use of a clamp and junior hacksaw. One quarter was ground in a pestle and mortar with liquid nitrogen, and the protein extracted from the powder. The remaining quarters were retained for later 2D protein gel analysis (not analysed due to humic acid interference). Bolus number 5 was freeze dried at -40°C for 5 days, before being ground to powder and sent to the IBERS Analytical Chemistry unit for proximate analysis for determination of dry matter and nitrogen content.

3.3.3. Vital stain of boli using Evans blue and damage analysis

As down boli were collected, boli were reserved for disassembly and subjection to vital stain 1% Evans blue dye. Disassembled boli were washed in H_2O to remove saliva, but all debris was retained within muslin before transferal of complete boli to staining vessel. Single boli were then stained for 10 min at room temperature with 1% Evans blue (in H_2O), excess stain was poured off and the samples rinsed three times in H_2O to remove remaining free Evans blue. Sub-samples of disassembled stained boli were then submerged in H_2O within a square petri dishes (number necessary to capture whole bolus) and photographed from a set distance (20.32 cm), with camera (14 MP) apparatus placed vertically against a consistent matt white back ground, within a known area and under controlled lighting from 2 sources (daylight bulbs to eliminate the effect of shadow, and promote true colour).

Three examples of wildtype and low PPO mutant boli were examined with basic RGB colourmetric analysis, with the blue and green/cyan regions isolated, and brown/white removed, to provide a very basic interpretation of percentage damage inflicted. This was performed using ImageJ (Rasband, 2015) designed to provide a quantitative analysis of five clusters (white, green, blue, cyan and brown) with pixel count (pixel count may also be converted to 2D area in ImageJ or MATLAB as distance from sample is known).

3.4. Protein extraction

Plant material was ground in a mortar pre-cooled with liquid nitrogen. To this, extraction buffer (0.1 M HEPES pH7.5 containing 2mM DTT, 1mMEDTA, 0.5% w/v protease inhibitor cocktail [Aprotinin, Bestatin, DMSO sol., E64, Leupeptin, Pepstatin A, PMSF, Sigma UK Ltd], and 0.1% Triton X100) was added at a ratio of 5ml/g fresh weight of sample, after grinding to powder and the material was further ground to a smooth homogenate, before been poured into individual pre-labelled 2 ml microfuge tubes. Samples were centrifuged for 10 min at -4°C at 10,000 xg to remove plant debris. The supernatant was then carefully removed using a 1 ml pipette into a second freshly labelled tube and aliquoted as required for further analysis and kept on ice.

Alternatively, protein was extracted by using the LE-LiDS (lithium extraction buffer) method (Mae *et al.* 1993) to remove proteins from tissue in an intact state. Plant material was ground in liquid nitrogen, in a mortar pre-cooled with liquid nitrogen, ice cold LE (1.7 ml orthophosphoric acid, 105 mg mono-iodoacetate, 25 g glycerol, 5 ml 2-mercaptoethanol, 85 mg phenylmethylsulfonyl fluoride [PMSF] dissolved in 5 ml methanol, pH adjusted to 7.2 in a final volume of 500 ml) was then applied (5 ml /g FW) and the material ground further with liquid nitrogen to powder. To this 0.5 ml /g FW of the detergent LiDS (20 g lithium dodecyl sulphate made up to 100ml of d. H_2O) was added and the sample ground again. The sample was transferred to pre-labelled 2ml microcentrifuge tubes, before being boiled for 2-5 minutes, and then centrifuged at 10,000 xg for 10 minutes. The resultant pellet was discarded, and the supernatant pipetted into a fresh pre-labelled Eppendorf for protein determination.

Where indicated 1% w/v insoluble PVP (polyvinylpyrrolidone), 40 mmol ascorbate or 1% w/v insoluble PVP plus 40 mmol ascorbate were added to extraction buffers at the initial grinding stage to prevent the action of PPO enzyme from affecting the results of protein determination. PVP was included as it was believed that it would act as a phenol scavenger and prevent quinone formation (Walter and Purcell, 1980), while the addition of ascorbate has been found to reduce protein breakdown over an extended time period when PPO is exposed to substrate through cell damage, acting as an antioxidant, depriving PPO of the oxidative element of its function (Winters and Minchin, 2001; Webb *et al.* 2014).

3.5. Biochemical assays

3.5.1. Protein determination

Quantitative protein analysis was performed using the Bradford protein assay (50 mg Coomassie blue, 50 ml methanol, 100 ml 85% w/v H_3PO_4, 500 ml H_2O, filter and add additional 350 ml H_2O; Bradford, 1976) using bovine serum albumin (BSA 1mg /ml) to generate standards. A standard curve of BSA protein in quantities of 0-20 µl was performed in triplicate (0-20 µl of BSA standard, 500-480 µl d. H_2O, 500 µl of Bradford reagent). Thereafter, to retain protein readings on the linear portion of the curve quantities of 2 µl plant protein samples were utilised (2 µl sample,

482 µl H_2O, 500 µl Bradford reagent). Control reactions containing aliquots of extraction buffers were also performed to calculate interference levels. All samples were inverted to fully mix contents within cuvettes, and were left undisturbed for 10 minutes before being read (with 1 ml d. H_2O as a blank) by spectrometer at 595 nm.

Quantitative protein was also measured by using a modified micro-Lowry protein assay (Lowry *et al.* 1951; increased accuracy to control values; Winters and Minchin, 2005), using BSA standards of 0-25 mg /ml. Reagents; four solutions (A, B_1, B_2 and C); A: 4.8% (w/v) Na_2CO_3 in 0.25% (w/v) NaOH, B_1: 2.4% (w/v) $CuSO_4 \cdot 5H_2O$, B_2: 4.8% (w/v) potassium sodium tartrate (stored at 4°C), C: 10 ml A + 0.1 ml B_1 + 0.1 ml B_2 and a diluted folin ciocalteu reagent (1 part folin reagent plus 8 parts H_2O). Aliquots of 50 µl of the aqueous sample was added to a round bottomed microtitre plate, together with 50 µl of Sol C, and incubated for 10 minutes at room temperature. After initial incubation, 50 µl of diluted folin reagent was added and the plate was incubated for a further 30 minutes. The absorbance was read at 700 nm and results calibrated with 0-25 µg BSA standard.

3.5.2. Determination of PPO activity

PPO was extracted, isolated and its activity measured in a method modified from that published previously (Robert *et al.* 1995; Winters and Minchin, 2001; Winters and Minchin, 2008). Protein extracts were desalted in columns prepared from desalting gel matrix Bio-Gel P6DG (BIO-RAD, 2003) prepared by resuspending 10 g; in 100 ml of McIlvaine buffer (176.5 ml 0.1 M citric acid and 823.5 ml 0.2 M sodium hydrogen phosphate). This was allowed to settle, and the top layer of McIlvaine buffer decanted, and replaced with a further 100 ml of McIlvaine buffer. This was repeated twice more over a 4 hr period to ensure total absorption of the buffer (Robert *et al.* 1995; Winters and Minchin, 2001; Winters and Minchin, 2008).

Desalting columns were prepared utilising 10 ml syringe barrels into which 2 discs (1 cm diameter) of micro-cloth filter were placed at the bottom of the syringe. The syringe was then inserted into a 50 ml screw cap tube (allowing for a 1 cm gap between the bottom of the tube and the syringe). Aliquots of 5ml of prepared P6DG were then pipetted into the syringe and the whole assembly was centrifuged at 10,000 xg for 6 minutes at 4°C. After centrifugation the collected

effluent was checked for any P6DG that would have indicated breakage of the micro-cloth, and then discarded, and the 50 ml tube was then cleaned and dried. The desalting columns were then set aside and covered while samples were prepared (Robert *et al.* 1995; Winters and Minchin, 2001; Winters and Minchin, 2008).

A mortar was cooled with liquid N_2 and pre-weighed subsamples of fresh, freeze dried or bolus material (as required) were crushed to powder with liquid N_2, before the addition of extraction buffer (0.5 g of ascorbic acid dissolved in 40ml of 0.2M sodium hydrogen phosphate, adjusted to pH7 with McIlvaine buffer (McIlvaine 1921) and maintained on ice) at 5 ml /mg FW, and re-ground as the homogenate thawed. The homogenate was then decanted into pre-labelled 2 ml microcentrifuge tubes, and centrifuged at 10,000 xg for 10 min at -4°C, after which the supernatant was decanted into a clean pre-labelled 2 ml microcentrifuge tube and retained on ice. An aliquot of 300 µl of the supernatant was then applied to the surface of the desalting column and allowed to settle for 1 minute before being spun for 6 minutes at -4°C at 10,000 xg. The PPO extract was then decanted into a clean pre-labelled 1.5 ml microcentrifuge tubes and retained on ice before use (Robert *et al.* 1995; Winters and Minchin, 2001; Winters and Minchin, 2008).

Plastic cuvettes were prepared in triplicate to contain 1.1 ml McIlvaine buffer pH 7.5, 15 µl of $CuSO_4$, and 20 µl of either SDS (to give total PPO activity) or H_2O (to determine the APPO pool) 375 µl of substrate (0.5ml of 0.1 M HCl, 19.5 ml of H_2O and 0.1 g of methylcatechol) and 20 µl of extracted enzyme. The contents were mixed briefly by pipette (3 intakes and expulsions), before immediately recording the change in absorbance at 420 nm, typically over a period of 30-60 sec. Resultant absorption graphs were then adjusted to give a linear regression, and the slope values were recorded (Robert *et al.* 1995; Winters and Minchin, 2001; Winters and Minchin, 2008).

Units of PPO activity (referred to as units /g DW) were calculated from absorbance curves over time, to $1\Delta AU_{420}$ /sec. where original absorbance of 30 sec was converted to absorbance over one minute ($AU._{420}$(60/30))=A, and converted to DM weight of sub sample (A*(1/0.05g))=B, and then converted to $AU._{420}$/sec (B/60), giving $1\Delta AU._{420}$/sec. These units of PPO activity were then used to compare PPO and APPO activity in all forage species.

3.5.3. Determination of bound phenol

Assay modified from Lowry (2.6.1 and Winters and Minchin, 2005), microplate capacity, pellet (as Lowry) extracted from 0.05 g freeze dried powder (grass species), 50 µl Lowry reagent A (0.45 M Na_2CO_3, 0.17 mM sodium potassium tartrate, in 0.14 NaOH) was mixed with 50 µl of sample in microplate well, while 50 µl of reagent A including 0.15 mM $CuSO_4$ was mixed with 50 µl of samples (fresh and mock masticated cocksfoot, perennial ryegrass and Timothy 2.4) in separate plate wells. Both plates were incubated for 10 min at room temperature, after which 50 µl of 1 N Folin-Ciocalteau reagent was added to each well and absorbances read at 650 nm on Bio-Rad Benchmark microplate reader after a further 30 min incubation. Standards of 0-0.5 mg ml^{-1} BSA were assayed simultaneously. Readings with and without copper were used to estimate protein and bound phenol concentrations (Winters and Minchin, 2005).

3.6. Protein gel electrophoresis

3.6.1. SDS PAGE

Aliquots from protein extracts prepared as described above (Section 2.6) were typically mixed with an equal volume of x2 protein loading buffer (made up by combining 4.7 ml H_2O, 1 ml 0.5 Tris, 1 ml glycerol, 1 ml 10% w/v SDS, 0.1 ml β-mercaptoethanol, 0.2 ml 0.05% w/v bromophenol blue; Laemmli, 1970) and boiled for 3 min in a water–bath. Where protein solutions were very dilute it was necessary to increase the concentration of the loading buffer such that 3+1 dilution of protein + loading buffer could be made.

Polypeptides contained in protein extracts were separated by SDS PAGE (BioRad Mini Protean II, used according to manufacturer's instructions; BioRad Ltd, Hemel Hemstead, UK). Single percentage separating gels were hand cast at either 12% w/v or 10% w/v acrylamide (12% w/v separating gel; 1.75 ml H_2O, 1.25 ml acrylomide/Bis 40% w/v [monomer], 1 ml SDS (3M Tris-HCl/SDS, pH 8.45) [running buffer], 0.004 ml tetramethylenediamine [TEMED] and 0.04 ml ammonium persulfate [APS]. 10%; 2 ml H_2O, 1 ml monomer, 1 ml running buffer, 0.004 ml TEMED

and 0.04 ml APS) with 4% stack at pH6.7 (1.3 ml H_2O, 0.2 ml monomer, 0.5 ml (3M Tris-HCl/SDS, pH 6.7) [stacking buffer], 0.003 ml TEMED and 0.02 ml APS).

Alternatively, where indicated precast 4-20% w/v gradient mini-PROTEAN gels (Bio-Rad) were used. Electrophoresis was performed for 45 minutes at 200V constant voltage, with the entire assembly (apart from power pack) located at 4°C .

Polypeptides were visualised by staining with Coomassie blue (500 ml methanol, 400ml H_2O, 100 ml acetic acid, 2.5 g Coomassie brilliant blue R-250 for 1 L of stain) (de St. Groth *et al.* 1963). Gels were placed in plastic trays, with enough stain to fully immerse the gel, and kept in motion on gel-shaker plate for 10-15 minutes to ensure total staining. After this time the stain was removed and the gels washed in H_2O before de-stain was applied (785 ml d. H_2O, 165 ml 100% ethanol w/v and 50 ml 100% acetic acid w/v for 1 L of de-stain) and the gel was returned to the gel-shaker for 20 minutes to 1 hr dependent on rate of de-staining. Gels were then washed with H_2O to remove de-stain and halt the process. Gels were retained in d. H_2O and scanned (BioRad GS800), after which gels were fixed in 7% w/v acetic acid, and stored at 4°C.

Where necessary, silver staining (Kerenyi and Gallyas, 1973) was used to visualise the bands formed after electrophoresis. This procedure is photochemical and was performed as following: reagents; silver solution – 0.1% $AgNO_3$ w/v in H_2O; developing solution – 3% w/v $NaCO_3$ in H_2O with 50 µl of formaldehyde/100ml (stir for 2 minutes). All incubations were conducted at room temperature, with continuous gentle agitation with all solutions mixed immediately prior to use. Sample gels were incubated for 15 minutes in 50% w/v MetOH. Sample gels were then incubated for 15 minutes in 5% w/v MetOH. a further incubation for 15 minutes in 32 µM DTT (8µl 1M DTT /250 ml water) solution, followed by two washes for 5-10 seconds with H_2O. Once washed, sample gels were then incubated with silver solution for 15 minutes. Silver stained gels were then washed twice for 5-10 seconds with H_2O and the developing solution was applied, incubation was then observed until sufficient band intensity had been resolved.

3.6.2. Western blotting

Abundance of specific polypeptides was determined by western blot techniques (Renart *et al.* 1979; Burnette 1981). After SDS PAGE gels were not stained but instead equilibrated in transfer

buffer (28.8 g glycine, 6.06 g Tris base and 400 ml w/v MetOH for 1L of buffer) for 15 minutes. Nitrocellulose (pre-cut to size of gel face, and clearly labelled) was moistened in H_2O, and retained in place within a Bio-Rad Trans BLOT assembly (BioRad Ltd, Hemel Hempstead, UK) according to manufacturer's instructions, ensuring no air bubbles or ripples were present. Electrophoresis was performed at 100 V for 1 hour to transfer polypeptides from gel to nitrocellulose. After this time transferring the nitrocellulose was carefully removed and placed in blocking buffer (Tris (pH 7.5), 3% w/v BSA and 0.1% w/v Tween 20) for 1 hour. Blots were then used for immunodetection of RuBisCo (LSU and SSU, diluted 1:5000 for 1 hr) according to Beha *et al.*, 2002 with alkaline phosphatase–conjugated rabbit immunoglobin used as the secondary antisera (diluted 1:2000 for a further hour), with three washes in TBS-T (1 ml Tween 20/L TBS) between each step. After incubations were complete blots were washed in TBS (pH 7.4; TBS-comprising 20 mM tris(hydroxymethyl)aminomethane (Tris) (HCL; pH 7.4; and 0.137 M NaCl) before being equilibrated with 100 mM diethanolamine buffer (pH9.8). Bands of cross-reaction were detected by incubation with alkaline phosphate reaction mixture (horseradish peroxidase) for 5-60 minutes at room temperature (dependent on rate of exposure). Once bands were clearly visible the reaction was terminated by placing the nitrocellulose in H_2O. Images were captured with GS-800 calibrated densitometer controlled via Quantity One 1-D Analysis Software (Bio-Rad Laboratories Ltd. Hemel Hempstead, UK).

3.7. Preparation and 2D proteomics

Two-dimensional analysis of polypeptide profiles of protein extracts was performed. Protein samples prepared as described above (section 2.6) and combined with equal part x2 gel loading buffer (section 2.7) were centrifuged at 98000 xg for 45 minutes at 4°C. The supernatant was removed to a clean tube, and the pellet re-suspended in 2ml extraction buffer. Protein samples were then centrifuged in clean tubes at 21000 xg for 10 minutes at 4°C until no pellet or material was left in the microcentrifuge tube. Supernatants were then precipitated by addition of 20% w/v TCA/acetone at 2:1 ratio. Samples were left at -20°C for >1 hr (samples inverted twice in first 40 minutes), before being centrifuged at 21000 xg for 15 minutes at 4°C to pellet precipitated protein. The supernatant was then removed via pipette, and 200 µl of ice cold 100% w/v acetone added. Protein pellets were resuspended by use of a sonicating water bath and collected by centrifuging at 21000 xg for 15 minutes at 4°C. This was repeated twice to remove any remaining TCA. After the final wash, acetone was removed via pipetting and drying at -20°C for 10-20 minutes to allow residual acetone to evaporate. Protein pellets were resuspended in 50 µl buffer Z (4.8 g 8 M urea,

200 mg CHAPS (8% w/v), 50 mg 33 mM DTT, 50µl ampholytes (pH 3-10, 0.5% w/v) (Morphew *et al.* 2007).

3.7.1. Mass spectrometry and protein identification

Protein quantification was performed by Bradford assay (section 2.6). Calculate quantity of protein sample in solution (µl) required for mass spectrometry and protein identification. Total protein sample (A) in extraction buffer (µl) / 100 = 1% of sample volume (µl). Percentage of sample required (150µg / total protein of µg A) = B. Volume of sample (µl) required for equal gel strip loading (A*B). Mixed with volume (µl) of buffer Z to created loading sample of 125 µl. An example of which is outlined below:

E.g. for 150 µl protein of a sample with 180 µg protein /ml sample:

(45/100) = 0.45

((150/180)*100) = 83.33%

83.33*0.45 = 37.5 µl of sample.

Add required volume of sample to enough buffer Z to make 125 µl (so 87.5 µl buffer) and mix.

Prepared sample volumes were applied evenly along the length of the well avoiding creation of bubbles, leaving 1.5 cm gaps at each end. Rehydration strips (7 cm, linear, pH3-10 IPG, Bio-Rad) were inserted along the sample well and paraffin oil pipetted to cover the strip until fully submerged. Samples were left over night (12 hours minimum) at room temperature (20°C). Focusing plates were prepared with pre-soaked (dd.H_2O) blotting paper strips. Rehydration strips were retrieved, and excess oil removed with absorbent paper. Rehydration strips were placed sample side down into the focusing plate, contacting the wick at each end. Rehydration strips were then submerged in 1ml paraffin oil, and the focusing plates closed. Focus plate assemblies were allowed to run at linear 40 mA, then 10000 V-hr for 3-4 hrs. Rehydration strips were then removed with forceps, excess oil blotted, and then placed sample side up in a rehydration tray and stored at -80°C until usage.

Handcast gels (12.5% w/v resolving gel/stacking gel, acrylamide (30% w/v) 4.21 ml / 0.375 ml, running gel buffer/stacking gel 2.5 ml/ 0.625, dd.H_2O 3.34 ml/ 1.5ml, ammonium persulphate (10% 0.1 g in 1 ml) 37.5 µl/ 12.5 µl, TEMED 10 µl/3.75 µl) with a layer of water saturated butanol. Gels were set after 30 min, water saturated layer was poured off and any residual washed with d.H_2O for both resolving and stacking gel steps.

Rehydration strips loaded with sample were equilibrated using the following process. DTT equilibration buffer (2 ml of; Tris base 1.5 M 181.5 g, dd.H_2O 750 ml, conc. HCl (µl) to achieve pH8.8, additional dd.H_2O to total sample volume of 1000 ml, stored at room temperature with DTT 20 mg/2 ml per resolving strip) was applied to the rehydration strips, and placed on a rocker for 15 minutes to reduce disulphide bridges of any proteins in the strip. DTT buffer was replaced with IAA buffer (2 ml; 50 mM Tris-Cl (6.7 ml 1.5 M solution), Urea 6 M 72.07 g, Glycerol 30% w/v 69.0 ml, SDS 2% w/v 4.0 g, bromophenol blue (small quantity, enough to colour solution), dd.H_2O up to 200 ml, store at -20°C and IAA 25 mg/2 ml p/resolving strip) and was left for 15 minutes on rocker, reducing disulphide bridges to prevent them from re-oxidising. IAA buffer was removed and rehydration strips could now be mounted on gels.

Protein-loaded rehydrated strips were mounted onto the hand cast gels, along with wicks loaded with 1 µl of protein marker. A solution of 0.5% w/v agarose was heated until fully molten, and pipetted carefully into the 1 cm gap at the top of the gel. Protein loaded rehydration strips were inserted centrally into the liquid agarose, and protein marker strips at either end before the agarose sets. Once set gels were run at 70 V until the dye front had crossed the stacking zone, then adjusted to 200 V until the dye front had reached the bottom of the gel.

Once complete, gels were stained using Coomassie brilliant blue stain for 30> minutes (Coomassie brilliant blue 2.5 g, methanol 100% 500 ml, acetic acid 100% 100 ml, made up to 1 L with H_2O) on the rocker. Gels were destained (40% methanol, 10% acetic acid 50% H_2O) on the rocker until sufficient Coomassie stain had been removed.

Gels were imaged via GS-800 calibrated densitometer (Bio-Rad). Imaged 2D gels were analysed using Progenesis PG220 v.2006 (nonlinear dynamics, Waters Company). Analysis was performed utilising non-spot background subtraction on average gels created from a minimum of three biological replicates. Spot volumes were normalised using total spot volume x total area. These were also used to determine any increase or decrease in protein abundance between

comparisons (with significance set at +2-fold change). Unmatched protein spots were detected between gel comparisons and protein spots (matched or unmatched) (Morphew *et al.* 2007) were excised using a scalpel, and sent for further identification (Dr Ian Brewis, Cardiff University) and analysis against the red clover protein map and also identified by using Mascot (Matrix Science). Further analysis of red clover protein using online databases; initial basic local alignment search tool BLAST analysis to further identify accession numbers from Mascot (BLAST, NCBI, registered trademark of the National Library of Medicine) and the UniProt Knowledgebase (UniProtKB, Uniprot consortium 2015) for further BLAST and or FASTA, alignment and ID mapping. Further identification, Signal P cleavage sites, pathway clarification, and comparison for conserved regions with; SignalP 4.1 Server (CBS Prediction Servers, Peterson *et al.* 2011), PROSITE scan, protein functional analysis (Sigrist *et al.* 2012), Clustal Omega (Goujon *et al.* 2010) multiple sequence alignment, Nucleic Acids Research Summary Database (Altschul *et al.* 1990) and KEGG metabolic pathways – reference pathway (Kanehisa *et al.* 2014).

3.8. Statistical methods

Statistical analyses were conducted using Genstat 13th Edition (VSN International 2011). Each separate analysis is described for in each experiment in its relevant chapter.

4. Results

The effect of PPO activity in grasses and red clover on the protein quality of down boli:

The effectiveness of PPO in protecting protein from proteolysis has been established in the context of silage making. During the ensiling process plant proteases (including peptidases) hydrolyse the majority of available plant protein to ammonia, free amino acids, and non-protein nitrogen, and as a result high levels of these compounds are considered good indicators of extensive proteolysis (Purwin *et al.* 2012[a]). However, in the presence of PPO phenolic compounds are converted to highly reactive quinones in the presence of oxygen (Parveen *et al.* 2010). Proteins are then rapidly bound by the quinones to form complexes that are more resistant to digestion by microbial or plant enzymes, resulting in lower levels of protein breakdown products being detected than in silage made from non-PPO containing forages (Winters *et al.* 2008; Lee *et al.* 2004; Broderick *et al.* 2001).

PPO activity can be rapid in the presence of oxygen, if sufficient oxygen is present during ingestive mastication then PPO-mediated protein complexing may occur while the forage is in the mouth and may continue as the bolus enters the rumen. Therefore, the primary aim of the research

described in this Chapter was to test the hypothesis that PPO activity could promote protein complexing in the timeframe of ingestive mastication. These experiments investigated the variation between three grass species with different reported endogenous levels of PPO activity, and between a wild type red clover and a mutant lacking PPO4 from the leaves (Webb *et al.* 2014) in terms of complexed protein, and the available protein during mastication and ingestion of grass boli.

Three experiments were designed to collect newly ingested forage down boli from four lactating Holstein × Friesian cows equipped with 100 mm diameter rumen cannula, utilising forages with different PPO content and activities. These experiments were coded and approved under the strict regulations described in the (1986) Animals (Scientific Procedures) Act. Procedure identifiers were TR217 (Red clover), TR211B (Grasses), experimental design details 2.3.1.

Activation of PPO was ~50% across species and treatments. After cutting and transport from the field, significant losses in PPO activity were observed for cocksfoot and Timothy but this was not observed in ryegrass. However, decreases in LPPO and APPO activity were detected in perennial ryegrass once it had been processed into the ingestive down boli. This decrease was not observed in cocksfoot in which the activity remaining in the grass at the farm site (< 15%) was retained after processing into the bolus (Fig 3.3).

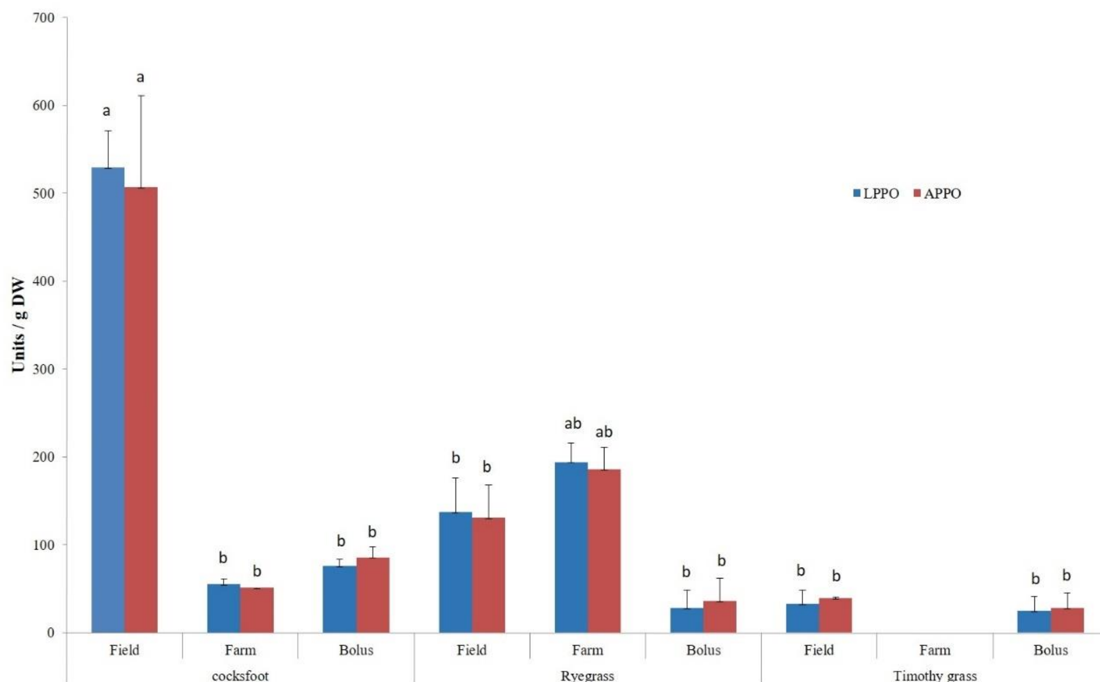

Figure 3.3 Effect of post-harvest transportation on PPO activity in zero grazed forage. PPO activity loss during cut and carry experiment where cocksfoot,

ryegrass and timothy (field) were harvested in the field, cut and transported (farm) and processed by animals to down boli (bolus). APPO = endogenous PPO activity without additional activation, LPPO = Latent PPO activity after SDS treatment to activate latent enzyme. Means ± sd of between three and six independent replicates are presented. Means without a common superscript letter are significantly different (P < 0.05) as analysed by two-way ANOVA and the TUKEY test.

The different grasses were analysed for DM, total N and protein content for different treatments. Significant differences in dry matter (DM) content were observed both between species and treatments for the grasses (Table 3.2). In field samples Timothy grass, cocksfoot and perennial ryegrass had dry matter contents comparable to that recorded in feed composition tables; cocksfoot 26%, perennial ryegrass 20% and Timothy grass 29% ∝ 28% and 32.6%, and 24-28.3% (Rodriguez-Carias, 2013; Limbourg and Lecomte, 1997). The %DM increased during the production of down boli (and during transportation for Timothy) by 17% and ~30% respectively. In ryegrass, a loss of ~12.6% DM was observed during the production of down boli. Nitrogen content showed significant variation between species and treatment (Table 3.2). There were significant (P<0.05) differences in recovered protein between species but not treatment (Table 3.2).

Table 3.2 Protein (mg/g DW), dry matter (DM; mg/g DW) and nitrogen content (N; mg/g DW) of cocksfoot, ryegrass and Timothy grasses. Data are representative samples collected from plots at Gogerddan (Field), after transportation to Trawscoed (Farm) and after collection as processed down boli (Bolus). Means resulting from between three and eight independent replicates are presented.

	Cocksfoot			Ryegrass			Timothy			*P*-value		
Variable	**FIELD** *n = 3*	**FARM** *n = 3*	**BOLUS** *n = 4*	**FIELD** *n = 3*	**FARM** *n = 3*	**BOLUS** *n = 8*	**FIELD** *n = 3*	**FARM** *n = 3*	**BOLUS** *n = 8*	**Species**	**Treatment**	**S×T**[1]
N	1.2 ± 0^{b}	1.2 ± 0^{ab}	1.5 ± 0.05^{a}	1.3 ± 0^{ab}	1.2 ± 0^{ab}	1.3 ± 0.04^{ab}	1.4 ± 0.05^{ab}	1.4 ± 0^{ab}	1.3 ± 0^{ab}	0.068	0.009	0.011
DM	26 ± 0^{bcd}	25 ± 0^{cd}	32 ± 1^{a}	20 ± 0^{e}	26 ± 0^{cd}	24 ± 0.31^{d}	29 ± 0.63^{ab}	28 ± 0^{ac}	27 ± 0^{bc}	<0.001	<0.001	<0.001
Protein	100 ± 14	100 ± 7.1	97 ± 7.1	120 ± 9.1	110 ± 15	100 ± 5	130 ± 12	120 ± 0.5	150 ± 45	0.04	0.493	0.935

Values are means ± SEM. $^{a-e}$Means in a row without a common superscript letter differ ($P < 0.01$) as analysed by two-way ANOVA and the TUKEY test. [1]S × T = Species × Treatment interaction effect. For protein no superscript is present as there is insignificant interference (field vs farm, field vs bolus, farm vs bolus) i.e. there is no significant difference between protein values between treatments.

The PPO activity of down boli was compared with that in field harvested samples (Fig 3.4). PPO assays confirmed that PPO activity was supressed in the mutant genotype, and that this could not be activated by the presence of SDS (Fig 3.4). As seen in the grass species, there were significant decreases in total PPO activity as a result of transportation (Fig 3.4). In the wild type red clover, the loss of the LPPO pool was slightly greater than the loss of the active pool (Fig 3.4). In the mutant the size of the LPPO pool was largely unaffected by transport but the active pool was significantly decreased to about 10% of field levels (Fig 3.4). Together this resulted in an alteration in the ratio of APPO: LPPO between the genotypes as a result of transport. These losses of PPO activity (<10% of original activity remained in the wild type, and <6% of original activity remained in the mutant) were observed despite minimal losses of protein (Fig 3.4 and Table 3.3).

There were no differences in %N between genotypes or a genotype*treatment interaction (Table 3.3). In the wild type there was no difference in %N from field to down boli (Table 3.3). However, in the mutant %N in the field differed from that presented at the farm and in down boli (Table 3.1). During transport from field to farm %N remained at comparable levels within genotypes. This difference seen in the mutant (in the field) resulted in a significant difference between treatments (P <0.001).

There were no significant differences in protein content between genotypes, or genotype*treatment (Table 3.3). Recovered protein for fresh material was ~10.7% for the wild type and ~10.3% for the mutant, with no significant difference. There were non-significant losses in protein between field to farm in the mutant (~10%) but not in the wild type. There were no significant differences in recovered protein between treatments (Table 3.3).

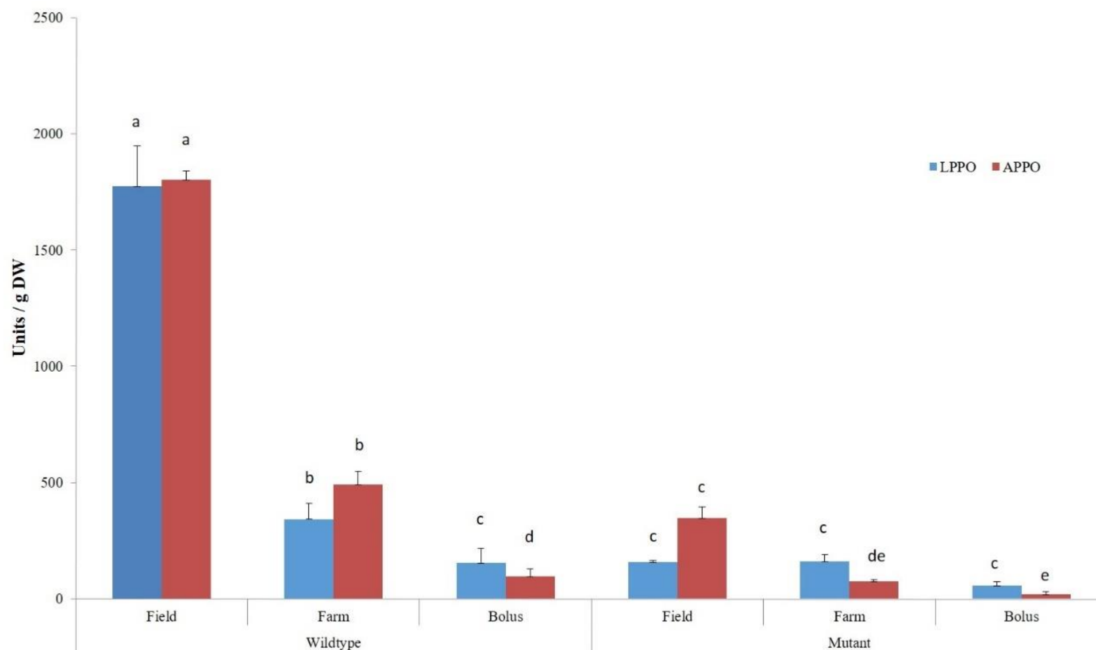

Figure 3.4 Effect of post-harvest transportation on PPO activity in zero grazed red clover. PPO activity loss was assessed during a cut and carry experiment where red clover wildtype (blue bars) and a low PPO mutant (red bars), were harvested in the field (field), cut and transported (farm) and processed by animals to down boli (bolus). APPO = Active endogenous PPO activity without additional activation, LPPO = Latent PPO activity after SDS treatment to activate latent enzyme. Means ± sd of between three and seven independent replicates are presented. [a-e]Means in a row without a common superscript letter differ (*P* < 0.05) as analysed by two-way ANOVA and the TUKEY test.

Table 3.3 Protein (mg/g DW), dry matter (mg/g DW) and nitrogen (mg/g DW) content of red clover wild type (cv Milvus) and the low PPO mutant. Data are representative samples collected from plots at Gogerddan (Field), after transportation to Trawscoed (Farm) and after collection as processed down boli (Bolus). Means of between three and eight independent replicates are presented.

Variable	Wild type			Mutant			*P*-value		
	FIELD	FARM	BOLUS	FIELD	FARM	BOLUS	Genotype	Treatment	G×T[1]
	($n = 3$)	($n = 3$)	($n = 8$)	($n = 3$)	($n = 3$)	($n = 8$)			
N	2.8 ± 0^{ac}	2.6 ± 0^{ac}	3.3 ± 0.13^{a}	1.9 ± 0^{c}	2.4 ± 0^{bc}	3.1 ± 0.14^{ab}	0.005	<0.001	0.074
DM	20 ± 0^{ab}	26 ± 0^{a}	16 ± 0.7^{bc}	23 ± 0^{a}	23 ± 0^{a}	14 ± 1.2^{c}	0.326	<0.001	0.048
Protein	100 ± 6.9	110 ± 6.9	77 ± 5	110 ± 3.4	94 ± 22	78 ± 9.8	0.847	0.029	0.739

Values are means ± SEM. [a-c]Means in a row without a common superscript letter are significantly different ($P < 0.01$) as analysed by two-way ANOVA and the TUKEY test. [1]G × T = Genotype × Treatment interaction effect. For protein no superscript is present as there is insignificant interference (field vs farm, field vs bolus, farm vs bolus) i.e. there is no significant difference between protein values between treatments

Down boli were stained with the vital stain Evans blue, photographed, and images analysed to determine the extent of blue dye retention (by dead and/or damaged cells) as a proportion of the total mass. Comparison of the Evans blue staining of three wild type and three mutant red clover boli revealed that a significant difference in damage by mastication occurred between the two genotypes (P< .01) with the animals inflicting ~fivefold more damage on wildtype red clover than the low PPO mutant (Table 3.4).

Table 3.4 Differential tissue damage in wild type and mutant red clover as a result of ingestion of fresh forage by four Holstein-Freisian rumen fistulated dairy cattle. Boli were deconstructed and stained in Evans blue dye to detect damaged cells. Images of boli were analysed for presence or absence of blue stain via the Pixel count function of ImageJ software, displayed as % blue pixels. Data are normally distributed and were subject to Two-tailed T-test.

Genotype	n	Mean	SD	t-cal	t-crit	df	p	Decision
Wildtype	3	20.0	4.56	6.17	5.6	4	.0035	P < .01
Mutant	3	3.7	0.46					

An assessment of the potential for PPO-mediated protein complexing in ingested and artificially created boli in a fresh forage feeding situation:

Experimentation was designed to assess the effect of high and low availability of PPO on protein complexing in freshly harvested red clover, processed as boli by cattle. Wild type red clover and the low PPO mutant were harvested at mid-morning from plots intended for silage at Cae Gwastad (Gogerddan) on 20th May 2011 and transported to the experimental cattle housed at Trawscoed (2.2, Fig 2.1). Plots were 200 m x 50 m and this was their second harvest for the year (plots were cut at 2 week intervals from May to October), plots had 2 m guard plots to separate them. Plants were harvested via Holdrup 1500 plot harvester (J. Haldrup a/s, Løgstør, Denmark) and cut to 5 cm above the soil to allow regrowth. Representative subsamples of plant material were immediately either flash-frozen in liquid nitrogen and stored at -80°C (at Gogerddan) or sampled and frozen at -20°C (at Trawscoed) for subsequent protein quantification and PPO activity measurement. The remainder of the forage was presented to animals which were fed either mutant or wild type red clover in a randomised cross over design (two periods). For each animal/feed combination the third fresh bolus was washed and retained for dry matter and nitrogen analysis while the subsequent boli (up to the 6th) were immediately frozen for later determination of protein, PPO and physical damage analysis.

It has been demonstrated previously that PPO activity can vary seasonally (Rees and Fothergill 2006). In order to confirm that the data presented in Chapter 3 (which was performed in

May 2011) was representative, the cut and carry experiment was repeated in June 2011 with PPO activity assessed at harvest and after transport to the farm.

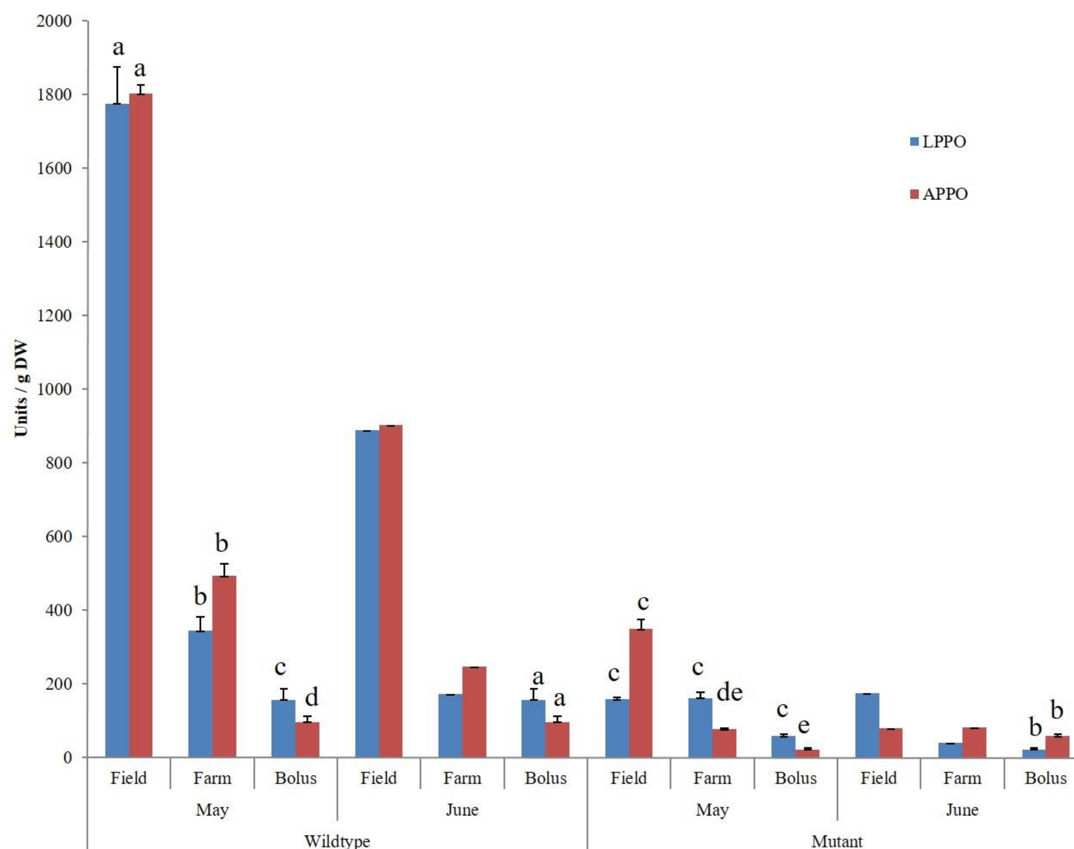

Figure 4.1 Comparison of PPO activity in field and as a result of transportation and bolus production for forages harvested in May and June 2011. APPO = Active endogenous PPO activity without additional activation, LPPO = Latent PPO, the activity only evident after SDS treatment to activate latent enzyme. Means ± SE are shown for n ≥ 3 independent replicates, apart from June field and farm where n=1. [a-e]Means without a common superscript letter differ (*P* < 0.05) as analysed by two-way ANOVA and the TUKEY test.

In both the May and June sample periods the percentage activation of wild type red clover PPO in the field was 50% (Fig 4.1). In the low PPO mutant red clover, the total activity (LPPO plus APPO) was approximately 17% of the wild type for May and 10% of the wild type in June. In the low PPO mutant, PPO was ~60% activated in May but was lower in June at ~30% (Fig 4.1). As the instability of the PPO enzyme during transport was a possible cause of loss of PPO activity, an experiment was designed to test the hypothesis that PPO activity could be preserved in the cut forage by altering transport conditions. Cold storage is used routinely in protein purification

protocols to decrease enzymatic activity, particularly proteases, or to decrease rates of physical decline, for instance of protein structure. Hence the effect of collection and transport at 4°C on PPO activity was compared with that after transport under ambient temperatures. In August 2011 wild type red clover was harvested from the site at Gogerddan and transported at either ambient temperatures or at 4°C (Fig 4.2.). It was notable that on this occasion the "field" samples collected under ambient conditions had similar activity to that recorded in June (Fig 4.1, 4.2), which increased to levels similar to those observed in May when harvested material was placed on ice for transport duration (~10-15 min). Transport under both ambient and cold conditions resulted in ~50% and ~80% loss of PPO activity respectively (Fig 4.2). Nevertheless, forage arriving at the farm having been cold transported had approximately twice the PPO activity of the comparable material that had been transported under ambient conditions, (Fig 4.2). Collection of samples following bolus production was limited by a clear refusal by the experimental animals to eat the ambient transported material, and a clear preference was observed for the cold transported material. This meant that animals consumed enough ambient transported material to produce just two boli that could be assessed for ambient transport as compared with a sample size of six for cold transported material.

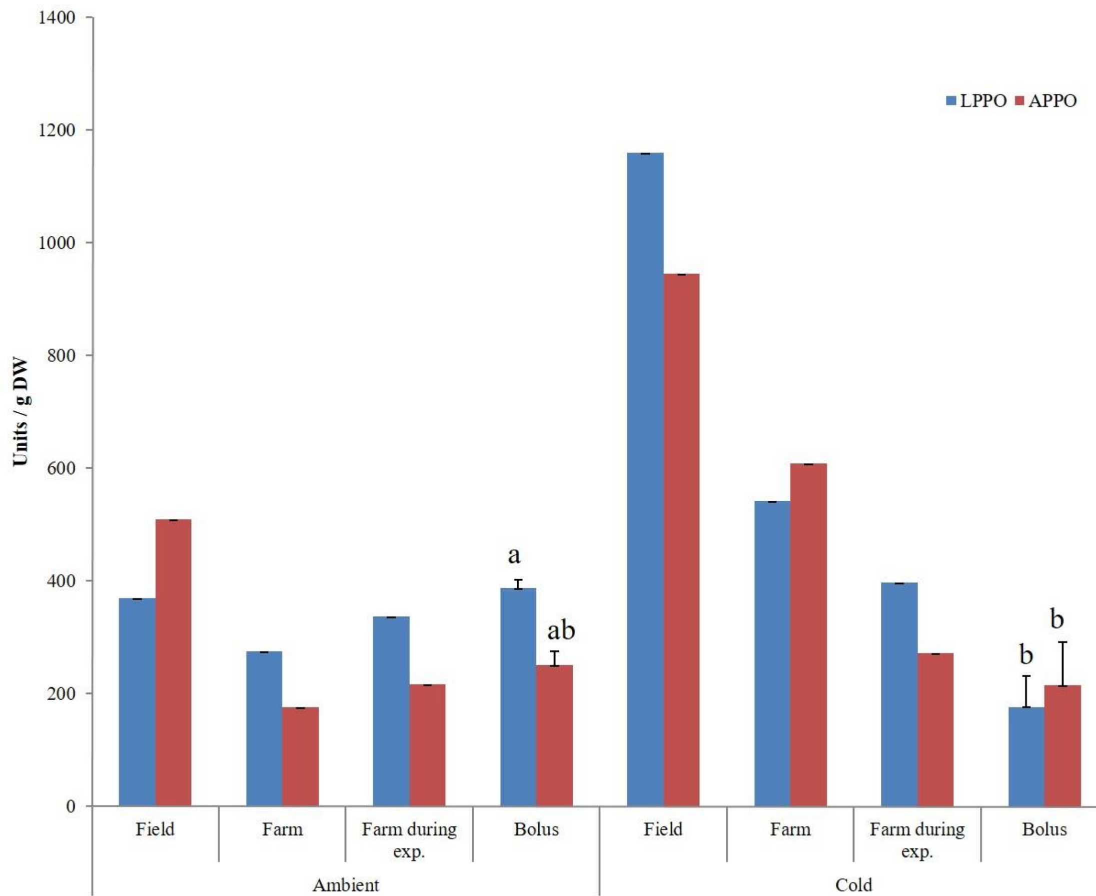

Figure 4.2 Effect of transport on wild type red clover harvested in the field (Field) and transported for 30min at either ambient or cold (-4°C) conditions to the farm (Farm) for use in feeding to produce down boli (Bolus). APPO = Active endogenous PPO activity without additional activation, LPPO = Latent PPO, the activity only evident after SDS treatment to activate latent enzyme. Means ± SE are shown for n ≥ 2 independent replicates, apart from field farm during exp. and farm where n=1. $^{a\text{-}b}$Means without a common superscript letter differ (*P* < 0.05) as analysed by two-way ANOVA and the TUKEY test.

In vitro assessment of anti-tryptic properties of PPO-mediated protein complexing:

To further understand the effect of PPO on cellular proteins and proteolysis a proteomic study was undertaken. Proteomics, the simultaneous analysis of the entire protein profile of a sample, offered an opportunity to identify specific proteins protected during complexing with PPO, and to examine the effect of such protection when subjected to tryptic digest. To examine which proteins were resistant to tryptic digest as a result of PPO activity, ascorbate was again used to inhibit PPO in red clover wild type plant samples generated where PPO was either active or inhibited, were separated by 2 dimensional gel electrophoresis (2D PAGE) which separates

polypeptides firstly on the basis of their isoelectric point and secondly by their mass/charge. The progression from SDS-PAGE gels to the use of two dimensions is useful in resolving individual peptides that may occupy the same or similar molecular mass. The successful identification of specifically protected proteins would be useful knowledge, especially in terms of plant breeding in order to increase levels of such proteins in red clover in order to magnify the protective effect, increase protein uptake of animals and reduce environmental impacts.

Initial investigations compared the protein profiles obtained when protein homogenates were allowed to complex for 30 min in either the presence (inactive PPO) or absence (APPO) of 40 mM ascorbate, followed by 40 min exposure to 1 unit of trypsin. Protein spots were observed for both treatments but the distribution throughout the gel was related to treatment (Fig 5.6). Where PPO was inhibited with ascorbate the majority of peptides were resolved at mid pH with molecular weights of less than 30 kDa (Fig 5.6.). Where PPO was active approximately 50% of the proteins were present in the acidic region of the gel with molecular weights up to 66 kDa (Fig 5.6.). The difference in profile between these two gels suggests that the higher molecular weight peptides from the minus ascorbate treatments are absent (or below detection limits) from the plus ascorbate treatments because these same proteins have been digested in PPO-inhibited samples. When the images were digitally combined a distinct grouping of polypeptides was visible in samples where PPO was active, of a size not present in PPO-inhibited samples (Fig 5.6.). These were the presumed unique proteins present in PPO-complexed material. Although theoretically the inclusion of ascorbate could have affected the running pH of the IEF gel this was considered to have been minimal due to the dilution and is confirmed by the retention of many "landmark spots" in the combined images.

Ascorbate is used in 2D electrophoresis to prevent the modification of proteins by phenolic compounds, with rapid precipitation (Geno Technology Inc., 2014). If ascorbate were to have an unmitigated effect on the pH of the sample or have an effect on the pH gradient of the 2D gel this could have resulted in two different profiles displayed. However, the inhibited and non-inhibited 2D gels presented (Fig 5.6) have many similarities, as can be seen in the red and blue gel overlay in the same pane, the differences in profiles appear to be proteins that are, possibly, prevented from

full migration by their absence from existing pools, due to the process of cell damage, extraction, and attempted digestion.

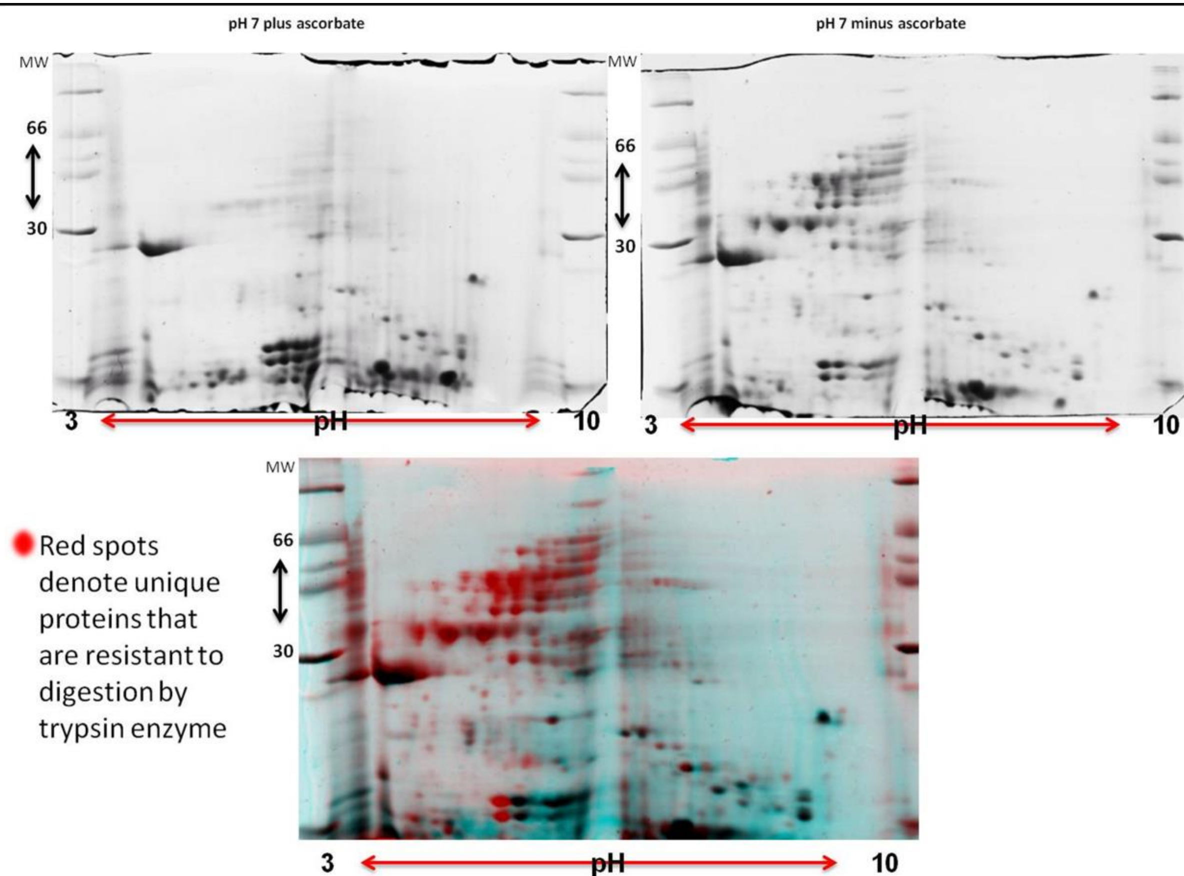

Figure 5.6 Proteome profiles obtained from red clover leaves ground at pH 7 (to simulate rumen) and allowed to form protein-quinone complexes (via PPO) over 30 min, then subjected to trypsin (at equal activity units) over 40 min. Samples were combined triplicate independent biological replicates which were run at equal protein loading (84 µg) on 2D gels. The coloured gel is a composite image of the 2D gels above.

Biological repeats (Appendix) were utilised to create two composite images (Fig 5.7-8) or gel maps displaying the placement of unique spots present under either treatment (with or without ascorbate prior to tryptic digest). However, variability in terms of both the overall number of distinct spots and their placement between the individual replicate 2D scans was detected. In non PPO-inhibited (minus ascorbate) samples ~63 distinct spots were observed, of which up to 13 were

unique to this treatment, while in the inhibited PPO samples (plus ascorbate) the number of spots did vary between replicates (51, 84 and 31 spots observed, of which six were unique).

Differences in spot distribution between treatments were investigated utilising Phoretics 2D Evolution software which removes much of the individual physical variance between gels, and allows greater accuracy to comparisons between treatments. By using this software, it was possible to identify spots which could be reliably assigned as unique to a treatment effect because they occurred on at least 3 gels within that treatment. Equalised gel images from this process were used to create gel maps (Fig 5.7 and 5.8.) which were then used to form a composite image for each treatment.

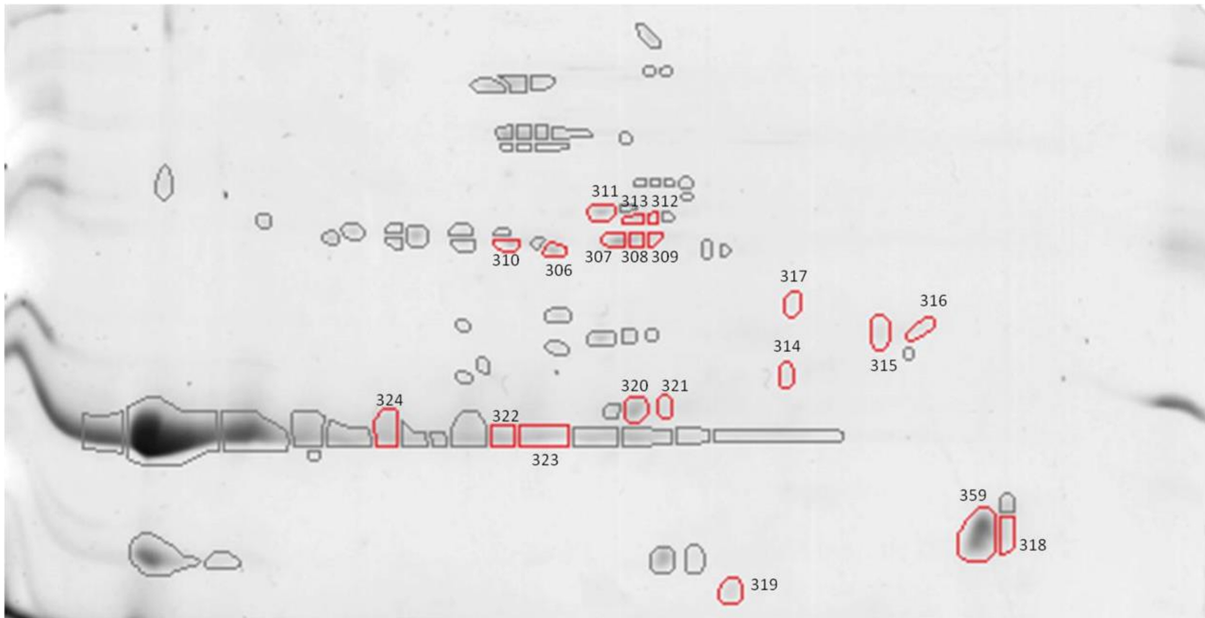

Figure 5.7 Composite gel map of unique spots present in red clover leaves extracted at pH 7 and allowed to form protein-quinone complexes (via PPO) for 30 min, before being subjected to trypsin (at equal activity units) for 40 min. Red areas depict unique spots present under this treatment in 3 or more replicates, spots 312, 319, 320 and 321 are unique to this treatment (Table 5.2). Samples were run at equal 84 µg on 2D gels (n=3).

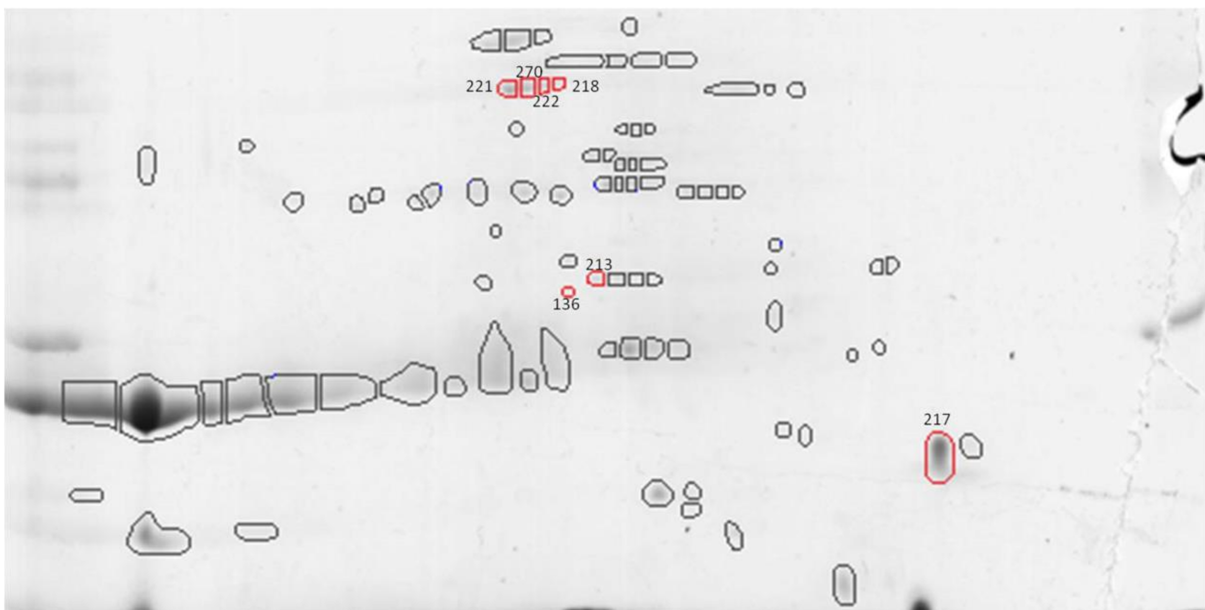

Figure 5.8 Composite gel Map of unique spots present in red clover leaves extracted at pH 7 with the inclusion of 40 mM ascorbate to inhibit PPO during the subsequent 30 min, then subjected to trypsin (at equal activity units) for 40 min. Red areas depict unique spots present under this treatment in three or more replicates. Samples were run at equal protein loading (84 µg 2D gels (n=3).

5. Industry messages

Red clover containing PPO can express different protein profiles to red clover with PPO inhibited and some of these proteins are resistant to tryptic digest. PPO may not be the direct cause of this effect. However, those proteins resistant to digestion are only present in red clover where PPO is not inhibited. Variability in PPO activity throughout the season, may also vary considerably dependent on environmental conditions, stress and the health of the crop. This makes experimentation on a small scale challenging but also has possible implications for the production of silage and free grazing. It may be possible that peaks and troughs of PPO activity may be predictable with more investigation, and that such knowledge may highlight the most productive time to crop, ensile or feed red clover. Cocksfoot grass remains a candidate for further investigation as a plant with naturally high PPO activity, though palatability may make investigation problematic. It remains to be seen whether PPO in the presence of oxygen, where plants have not been stressed, could indeed have a protective effect – or to at least form the complexed-hard to digest complexed-proteins. However, the potential may be there and it may be worth further investigation, with a strategy to ensure plants are in the best condition, and to compare effects with those seen in silage more directly.

6. Discussion

Since PPO was first identified in plants (Yoshida, 1883), the focus in terms of gaining knowledge about the enzyme has been on red clover, because of its role in ensiling and its potential for cloning in alfalfa (Sullivan *et al.* 2004; Lee *et al.* 2004; Thipyapong *et al.* 2007; Winters *et al.* 2008; Lee *et al.* 2009; Eickler *et al.* 2011) and other plants. In this research PPO from the leaves and stems of red clover and grass species were extracted and analysed, in terms of activity and the possible effect of the presence of the enzyme on plant protein. There was no direct evidence of the formation of protein-quinone complexes within these experiments, which have been observed previously (Winters *et al.* 2008). This is intriguing in itself, as it suggests that the activity, or activity pool of PPO (plus conversion and reversion from LPPO to APPO) may be a more complex issue than previously observed. Previous research with red clover silage (Lee, 2004) has observed the protection of proteins through a PPO mechanism, that allows greater uptake of protein in the small intestine. This may suggest an investigable difference between the activity of PPO and/or availability of substrate between fresh and ensiled material. If this difference can be reduced to a component treatment, stressor, or environmental condition etc. it may be possible to create a

similar instance in fresh material offered to experimental animals.

Theoretically, the window of opportunity where PPO, oxygen and phenolic substrates are all present (within the mouth) is small, but does exist. The rapidity of PPO activation and activity has already been observed in this and previous research. It may be beneficial in future research involving these plant species, as well as others containing PPO, to attempt to track the potential progress of protein complexing (or lack thereof) by investigating the presence of quinones within molecular weights of protein (SDS PAGE) in both fresh and ensiled material. The presence or absence of which could more accurately indicated which proteins are most likely to be the subject of interaction with PPO.

It is possible that in the experiments recorded here, where plants were not subject to good growth conditions, usual weather patterns, lack of rain fall, predation etc. that may have resulted in dehydration causing a degradation of PPO within plants before and possibly after harvest. This may explain the low levels of complexing observed, despite the same opportunity to do so when compared to existing research. The suggested mechanism of PPO degradation is a Fenton-like reaction, where H_2O_2 plus Cu can yield HO radical; and the addition of ascorbate to this process is necessary to prevent the formation of reactive oxygen species (ROS) (Kuwabara and Katoh, 1999). Such degradation could have occurred as a result of hydroxyl radicals generated in a Fenton-type reaction with the endogenous Cu. ROS can physically damage proteins and DNA (Kingston-Smith and Foyer, 2000). Assuming sufficient temperature and dehydration were experienced to cause levels of hydroxyl radicals to be raised within the chloroplast, this would result in oxidation of proteins including PPO, membrane damage would permit cytoplasmic proteasomes or vacuolar proteases access to the damaged proteins for degradation, resulting in loss of protein and less PPO activity compared with previous findings (Winters *et al.* 2003; Winters *et al.* 2008; Lee *et al.* 2006). This is a testable hypothesis, as the extent of carbonylation of PPO protein (Kingston-Smith and Foyer, 2000) and measuring the antioxidant and hydrogen peroxide status of plant tissue, after growth and harvest in warm, dry conditions.

A resolution for these issues associated with abiotic stress was investigated by sowing red clover and grass species with 20m of the experimental animal site. Poor growth conditions, pest and mechanical damage unfortunately delayed, and ultimately resulted in small/poor quality yields. Other methods for future consideration were growth trays, either soil based or hydroponic, where plant material with PPO content, can be more closely managed, and offered without directly harvesting (especially hydroponically where the whole plant can be offered).

Alternatively, further investigation into the mechanism by which PPO enzyme and/or activity is lost could be achieved by a comparison of effects on another labile enzyme, such as aureusidin synthase (Nakayama, 2000). This enzyme is a PPO homolog responsible for flower colouration, usually in plants with yellow flowers. Aureusidin synthase has already been transgenically inserted into leaves to alter leaf colour (Shakya *et al.* 2012) and like PPO, contains Cu. Aureusidin synthase resides in the cytoplasm (unlike PPO in within the chloroplast) (Thipyapong *et al.* 2007), and so its

activity could be determined alongside that of PPO during cut and carry experiments. The performance of the inserted enzyme in comparison to that of PPO could help confirm any actual difference in enzymatic activity within the chloroplast, or whether this was an artefact of the investigation.

In red clover protein losses observed in the production of down boli (with little variation in %N and %DM) were also undesirable, and may represent a proportion of physically lost protein. This result may also indicate changes in protein quality in terms of relative proportions of individual proteins, again feed quality may have been a factor of this loss (large losses in PPO activity may also represent a portion of lost protein). However, even minimising such relatively small losses in protein could be important in terms of the potential feed return of possible PPO-mediated protein protection, and the cost saving of N return to farm land, may make such small losses acceptable (Genever *et al.* 2013).

During the investigation of attempting to identify specific protected protein, plant cell contents were homogenised and so PPO was free to create quinones, which could then have potentially reacted with any one of hundreds of potential proteins. It was therefore surprising that only four proteins were identified as being altered in abundance as a result of the presence of PPO (and the absence of ascorbate), and that they were all chloroplastic. Although all four were co-located in the thylakoid membrane as is PPO, there was no evidence that the proteins were mechanistically linked to PPO. There has been considerable debate over the reason for localisation of PPO in the thylakoid and although various theories have been proposed in favour of a link between PPO and photosynthesis evidence is still lacking (Boeckx *et al.*, 2015[a]). It was therefore possible that these proteins are similar in terms of their composition and/or structure. Although as only a small region of sequence homology (three amino acids) was detected this may indicate other similarities that could potentially be exploited, such as the tertiary structure. Further studies could be designed to determine if these four target proteins are specific to trypsin, with the use of other proteolytic enzymes such as papain, α-chymotrypsin and pepsin which target different sequences. Further, protein stability in the presence of proteases isolated from livestock, and specifically proteolytic enzymes from ruminants, should be assessed, along with the potential to increase the abundance of such naturally resistant proteins within suitable plant species.

To conclude;

- The presence of functional PPO enzyme pre-digestion (by trypsin) did not create stable protein-quinone complexes *in vitro*, a small group of chloroplastic transit peptides (proteins) were retained, but this may not be related to PPO activity.
- PPO enzyme is a more complex enzyme than recorded in previous research, in terms of reaction to temperature, season, dehydration, early fermentation and transport after harvest.
- There are practical limitations to the exploitation of PPO in red clover to protect protein delivered to ruminants in fresh forage.
- Large quantities of PPO in red clover or grass species do not necessarily equate to proportional activity potential at the point of delivery to the animal.
- Differences in isoform(s) of PPO between plant species may be more important in the future identification of useful PPO to encourage the potential for a protein-protective effect.
- The activation of PPO (up to ~50% across species) at harvest was not retained after transport resulting in near total loss of activity in down boli.
- The identification of chloroplastic proteins which may have an unconfirmed role associated with PPO (either in the transit of the mature protein, or as activated by enzymatic activity) before significant damage occurs to the cell, also adds to the knowledge described by others regarding the function of plant proteins during autolysis (Kingston-Smith *et al.* 2008; Beha *et al.* 2002; Lee, 2014).

A combination of a minimal time frame of available O_2 and damage dependent availability of substrate are less amenable to manipulation than increasing the proportion of APPO available during the formation of the down boli. Further work will be required into understanding the properties of PPO in red clover, including control of the activation state. A logical step for further *in vitro* investigation would seem to be a minute by minute sampling of complexed protein under controlled O_2, rumen temperature and pH conditions; this would provide a very clear viewpoint into the specific window of opportunity available in the formation of down boli.

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7.1. Abbreviations

#1403 Animal identification number

#3055 Animal identification number

#5391 Animal identification number

#6343 Animal identification number

1D One-dimensional

2D Two-dimensional

AberDart Lolium perenne cv. AberDart (Perennial ryegrass)

AberDovey Festuca arundinacea cv. Dovey

AberEpic	Lolium multiflorum cv. AberEpic
AberMagic	Lolium perenne cv. AberMagic
AberTop	Dactylis glomerata cv. AberTop (Cocksfoot)
Ad Lib	Ad libitum
ANOVA	Analysis of Variance
APPO	Active PPO
BLAST	Basic local alignment search tool
Bolus	Mass of chewed food at moment of swallowing
Boli	Plural of bolus
Cae Gwastad	Field location of Red clover and low PPO mutant
Cae Penlon	Field location Trawscoed research farm of forages
CHAPS	3-[(3-Chloramidopropyl)dimethylammonio]-1-propanesulphonate
CP	Crude protein
cTP	Chloroplastic transit peptide
d. H2O	Distilled water
dd. H2O	Double distilled water
DEFRA	Department for Environment, Food and Rural Affairs
DM	Dry matter
Down boli	Initial swallow (by cow) of plant material, caught rumen entrance
DTi	Department of Trade and Industry
DTi	Development of trade and industry
DTT	Dithiothreitol
DW	Dry weight
EBLEX	English Beef and Lamb Executive Ltd.
EPSRC	Engineering and Physical Sciences Research Council
EPSRC	Engineering and Physical Sciences Research Council
FASTA	Fast-all (fast protein comparison)
FW	Fresh weight
GHG	Greenhouse gas

ha	Hectare
HCC	Hybu Cig Cymru
HCl	Hydrochloric acid
Holdrup	Holdrup 1500 plot harvester
HPLC	High-performance Liquid Chromatography
IAA	Iodoacetamide
ImageJ	Open source scientific multidimensional image processing
K2O	Potassium oxide
kDa	Kilodaltons
kDa	Kilodaltons (molecular weight)
KEGG	Kyoto Encyclopaedia of Genes and Genomes
Low PPO	T. pratense cv. Milvus mutant expressing greatly reduced levels of PPO
	Polyphenol oxidase (enzyme)
LPPO	Latent PPO
LSU	large sub-unit
m	metres
MATLAB	Matrix laboratory
MetOH	Methanol
N	Nitrogen
N terminus	Amino or NH2 terminus
NCBI	National Centre for Biotechnology Information
P2O5	Phosphoric anhydride
PMSF	Phenylmethylsulfonyl fluoride
PPO	Polyphenol oxidase
PPO1	Found in red clover leaves
PPO2	Red clover flowers and petioles
PPO3	Broadly over leaves, possibly flowers
PROSITE	Protein database
PUFA	Polyunsaturated fatty acids

QMS	Quality Meat Scotland
Red clover	*Trifolium pratense* cv. Milvus
RGB	Red, green, blue
ROS	Reactive Oxygen Species
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S.48	*Phleum pratense* L. cv s48 (Timothy grass)
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERF	Succinic esters from renewable feedstocks
SSU	Small sub-unit
TCA	Trichloroacetic acid
TEMED	Tetramethylenediamine
TR211	Grass forage animal experiment 1
TR211B	Modified version of TR211 (experiment 2)
TR217	Red clover and low PPO red clover animal experiment (experiment 3)
Tris base	Trisaminomethane
Units	Units of PPO activity calculated from absorbance curves 1ΔAU420/sec
V-hr	Volt-hour
w/v	Weight/volume
ΔOD	Absorbance