JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 6611–6617 6611
DOI:10.1021/jf900808p

In Vitro Study of Red Clover Polyphenol Oxidase Activity, Activation, and Effect on Measured Lipase Activity and Lipolysis

G. Van Ranst,[†] V. Fievez,*[,‡] M. Vandewalle,[†] J. De Riek,[†] and E. Van Bockstaele[†,§]

[†]Institute for Agriculture and Fisheries Research, Unit Plant, Applied Genetics and Breeding, Caritasstraat 21, 9090 Melle, Belgium, [‡]Ghent University, LANUPRO, Proefhoevestraat 10, 9090 Melle, Belgium, and [§]Ghent University, Plant Production, Coupure Links 653, 9000 Ghent, Belgium

The goal of this paper was, first, to study the effect of red clover polyphenol oxidase (PPO) activity on protein-bound phenols and measured lipase activity *in vitro* and, second, to study the effect of PPO activation, measured as an increase in protein-bound phenols, as a result of degrees of damaging (not damaged, crushed, and freeze/thawed) of red clover before wilting on measured enzyme activity and *in vitro* lipid metabolism when incubated in a phosphate buffer. There was a positive relation between PPO activity and the occurrence of protein-bound phenols with a concomitant decrease in measured lipase activity, indicating a possibility to a direct inhibition of enzymes as a result of protein-bound phenols. Furthermore, damaging can activate PPO in red clover, measured as an increase in protein-bound phenols during wilting [0.7−20.6 nmol of tyrosine equiv (mg of protein)$^{-1}$], again with a concomitant decrease in measured lipase activity [41.3−20.3 μmol of *p*-nitrophenyl butyrate (PNPB) min^{-1} (mg of protein)$^{-1}$]. Lipid metabolism during incubation of these forages in a phosphate buffer with ascorbic acid was only influenced by damaging when wilted for 24 h, with a lower lipolysis in crushed and freeze/thawed (52.9 and 32.6%, respectively, after 8 h of incubation) material compared to all other treatments (on average 60.4% after 8 h of incubation).

KEYWORDS: Silage; fatty acids; lipid metabolism; red clover; polyphenol oxidase

INTRODUCTION

Polyphenol oxidase (PPO) is a phenol and diphenol oxidizing enzyme, especially active in red clover more so than in other forages (*1*). Because of this oxidation, quinones are formed, which are reactive molecules capable of binding to nucleophilic binding sites, e.g., some amino acids. Therefore, these quinones can bind to proteins. The influence of red clover PPO on proteolysis and lipolysis, two important degradation processes in silages, has been studied *in vitro* and *in silo* (*2*−*5*). This showed that proteolysis as well as lipolysis can be inhibited in forages with PPO activity. A reduced *in silo* lipolysis might be of interest because this could be hypothesized to reduce the consecutive biohydrogenation of the polyunsaturated fatty acids (PUFAs) in the rumen (*6*). The latter is desirable because of health claims on PUFAs, which are generally low in ruminant products. There are two hypotheses how PPO could inhibit lipolysis: (1) direct enzyme inhibition as a result of binding of quinones to the enzymes and thus blocking the substrate binding site and (2) substrate protection as a result of binding of the quinones to the substrate and/or encapsulation of the substrate in proteins with bound quinones. PPO in a living plant is present in a latent form, separated from its substrates (phenols). Therefore, to become active, plant stress (e.g., damaging) is required (*7*). Furthermore, the time needed after activation to allow sufficient PPO-mediated diphenol oxidation followed by binding to proteins to induce a significant decrease in lipase activity and lipolysis, is not clear.

The first goal of this study was to evaluate the possibility of direct lipase inhibition as a result of protein-bound phenols, induced by PPO activity, without, however, excluding the option of an indirect inhibition by protection of the lipids as a result of bound phenols (experiment 1). The second goal was to study the effect of damaging and wilting time on PPO activation, assessed by the increase in protein-bound phenols, as well as on lipase activity and *in vitro* lipid metabolism (experiment 2).

MATERIALS AND METHODS

Experiment 1. For the first experiment, one protein extract of red clover cv. Milvus was used (location: 50° 59′ 5″ N/3° 46′ 27″ E). The red clover was harvested during late autumn of 2008 using scissors. The protein extract was made as described by Van Ranst et al. (*6*). Briefly, after fresh plant material was ground in liquid nitrogen, approximately 42 g of the fresh plant material was extracted with 280 mL of phosphate buffer with 30 mM ascorbic acid (pH 7.0). Proteins were precipitated using ice-cold acetone, and the pellet, recovered after centrifugation, was redissolved in 42 mL of phosphate buffer without ascorbic acid (pH 7.0). Using this single protein extract, all measurements were performed in triplicate. In an aliquot of 3 mL of this protein solution, 0.5 mL of 4-methyl-catechol (0.2 M; Sigma, Bornem, Belgium) was added. This mixture was incubated

*To whom correspondence should be addressed. E-mail: veerle.fievez@ugent.be.

Published on Web 07/02/2009

for 0, 10, 20, 60, or 120 s. The PPO activity in the extract was varied through different concentrations of NaCN (Sigma, Bornem, Belgium) in the incubation buffer, namely, 0, 0.1, or 5.0 mM. A separate aliquot of the original protein solution was used for each time point and NaCN concentration. Incubation was stopped by adding four volumes of ice-cold acetone. The proteins were allowed to precipitate for 35 min at −20 °C. After centrifugation (3500*g*, 10 min, 4 °C; Beckman J2-HS with JA-17 123 rotor, GMI, Inc., MN), the supernatant was discarded and protein and protein-bound phenols were determined in the pellet according to Winters and Minchin (*8*), which is adjusted from the spectrophotometrical Lowry method for protein determination using the Folin−Ciocalteau reagent (*9*). Lipase activity in the pellet was measured spectrophotometrically (400 nm) using *para*-nitrophenylbutyrate (PNPB) as a substrate according to Van Ranst et al. (*6*).

Experiment 2. For the second experiment, red clover cv. Lemmon was sown in three replicates on Sept 12, 2006 and mowed on five occasions in 2007 (location: 50° 59′ 5″ N/3° 46′ 27″ E). The cut used for this experiment was harvested on Aug 5, 2008 using an Haldrup harvester (J. Haldrup s/a, Løgstør, Denmark). This was the third cut of 2008; the two former cuts were taken on May 19, 2008 and June 30, 2008. The three replicates in the field were kept as replicates throughout the experiment. A sample was taken of the fresh red clover; after which, the forages were separated into three parts. One part was not damaged; a second was damaged by crushing by hand (squeezing and turning); and a third part was quickly frozen using liquid nitrogen and thawed at room temperature (freeze/thawed). After the sample was damaged, the forages were left on the floor at approximately 16 °C for 4 or 24 h before sampling. Samples were immediately frozen in liquid nitrogen, ground in a mortar with liquid nitrogen, and stored at −80 °C until analysis. Directly on the sampled forages, proteins, protein-bound phenols (*8*), PPO (*10*), and lipase activity (*6*) were measured. Approximately 3 g of sample was incubated in a phosphate buffer (0.1 M; pH 7.2) with 30 mM ascorbic acid for 0, 1, 2, 4, and 8 h. Ascorbic acid was added to the incubation buffer to avoid PPO activity during the incubation, to measure the effect of PPO activity during wilting rather than during the incubation. After this incubation, a fatty acid (FA) extract was made using chloroform/methanol (2:1, v/v) (*11*) and 10 mg of C19:0 (Sigma, Bornem, Belgium) was added as an internal standard. Lipids were separated into free fatty acids (FFAs), membrane lipids (MLs), and neutral fraction [triacylglycerols (TAGs), diacylglycerols (DAGs), and monoacylglycerols] using two kinds of solid-phase extraction (SPE) columns (*12*). To these three fractions, 10 mg of internal standard (C21:0) was added before methylation (*13*). The total FA and FA in the lipid fractions were analyzed after methylation and gas chromatography (GC; Hewlett-Packard 6890 gas chromatograph, Hewlett-Packard, Brussels, Belgium) with a Solgel-wax column (30 m × 0.25 mm × 0.25 μm; SGE Analytical Science, Victoria, Australia) and a flame ionization detector (FID). The temperature program was as follows: 150 °C for 2 min; increased at 3 °C min^{-1} until 250 °C; injector temperature, 250 °C; and detector temperature, 280 °C. The FA content was calculated using the area of the internal standard as a reference, taking into account the theoretical response factor. Lipolysis was calculated as the difference in proportion of total FA that was occurring in the ML between the 0 and 1, 2, 4, or 8 h of incubation, respectively, and divided by the proportion of total FA in the ML after 0 h of incubation.

Statistics. All results were analyzed using the general linear models of SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL). Multiple comparisons were performed using the Tukey test. For the incubations in experiment 1, the following model was used: $Y_{ij} = \mu + \mathrm{NaCN}_{i=1,\ldots,3} + I_{j=1,\ldots,5} + \varepsilon_{ij}$, with NaCN being the NaCN concentration in the incubation buffer (0, 0.1, or 5.0 mM) and *I* being the effect of the incubation time (0, 10, 20, 60, or 120 s). Two-way interaction between the main effects was tested in the same model. For the incubation in experiment 2, the following model was used: $Y_{ijk} = \mu + W_{i=1,\ldots,3} + T_{j=1,\ldots,4} + I_{k=1,\ldots,5} + \varepsilon_{ijk}$, with fixed factors *W* being the effect of wilting (0, 4, or 24 h), *T* being the effect of treatment (fresh, no damage, crushing, and freeze/thawing), and *I* being the effect of the incubation time (0, 1, 2, 4, or 8 h) as a repeated measure. Two- and three-way interactions were tested in the same model.

RESULTS

Experiment 1. The PPO activity was measured in a phosphate buffer (pH 7.0) with a range of NaCN concentrations. Incubations with 0.1 mM NaCN showed 74.8 ± 14.7% of the control PPO activity, as measured in the incubation without NaCN. When 5.0 mM NaCN was added to the phosphate buffer, only 1.8 ±0.03% of the activity was left.

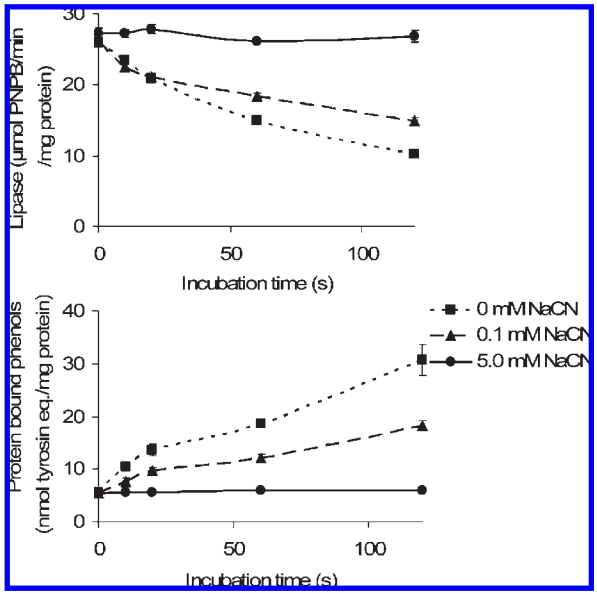

Figure 1. Influence of the NaCN concentration in the incubation buffer of a protein extract of red clover with 4-methyl-cathecol on measured lipase activity [μmol of PNPB min^{-1} (mg of protein)$^{-1}$] and protein-bound phenols [nmol of tyrosine equiv (mg of protein)$^{-1}$]. The error bars indicate the standard error of the mean (n = 3).

Table 1. PPO [ΔA min^{-1} (mg of Protein)$^{-1}$], Lipase Activity [μmol of PNPB min^{-1} (mg of Protein)$^{-1}$], and Protein-Bound Phenols [nmol of Tyrosine equiv (mg of Protein)$^{-1}$] in a Protein Extract of Either Fresh, Not Damaged, Crushed, or FT Red Clover[a]

		PPO activity [ΔA min^{-1} (mg of protein)$^{-1}$]		lipase activity [μmol of PNPB min^{-1} (mg of protein)$^{-1}$]		protein-bound phenols [nmol of tyrosin equiv (mg of protein)$^{-1}$]	
treatment	wilting time (h)	mean	SEM[b]	mean	SEM	mean	SEM
fresh	0	16.1 a,b	1.57	41.3 a,b	1.97	0.746 d	0.3606
no damage	4	26.7 a	0.92	41.0 a	2.54	0.874 d	0.4295
	24	19.2 a,b	2.35	34.8 a,c	2.14	2.19 c,d	0.448
crushed	4	16.7 a,b	3.42	28.1 b,c	2.41	4.77 c	0.349
	24	20.0 a,b	5.45	21.2 b	3.22	7.38 b	0.617
FT	4	11.5 b	0.87	24.2 b,c	0.54	9.69 b	1.893
	24	13.3 a,b	2.83	20.3 b	2.92	20.6 a	2.31

[a] All treatments except for the fresh were wilted for 4 and 24 h (n = 3). Different letters indicate a significant difference (p < 0.05) between treatments and wilting times. [b] SEM = Standard error of the mean.

In **Figure 1**, the results of the lipase activity and the protein-bound phenol measurements are given. Therefore, a protein extract of red clover was incubated with 4-methyl-catechol as a substrate for PPO. Different NaCN concentrations were applied to induce a variation in the PPO activity. The duration of the incubations was 0, 10, 20, 60, or 120 s. Lipase activity decreased from 26.1 to 10.2 and from 26.1 to 14.9 μmol of PNPB min^{-1} (mg of protein)$^{-1}$ during the incubation when no or 0.1 mM NaCN was applied, respectively. At the highest NaCN concentration, lipase activity was not affected by the 2 min incubations.

Concomitantly, the bound phenols were influenced by NaCN, with an increase in the incubations without NaCN from 5.5 to 30.7 nmol of tyrosine equiv (mg of protein)$^{-1}$, from 5.4 to 18.3 nmol of tyrosine equiv (mg of protein)$^{-1}$, and no effect of the incubation time with the highest concentration of NaCN in the buffer.

Experiment 2. In the first part of this experiment, PPO, lipase activity, and protein-bound phenols in a protein extract of red clover were measured. Before making the extract, red clover was treated in different ways: (1) no damaging, (2) crushing, or (3) quickly freezing with liquid nitrogen followed by thawing. The results are presented in **Table 1**. PPO activity varied considerably between treatments and wilting times, being highest after 4 h of wilting without damaging [26.7 ΔA min^{-1} (mg of protein)$^{-1}$] and lowest after 4 h of wilting with previous freeze/thawing (FT) [11.5 ΔA min^{-1} (mg of protein)$^{-1}$]. When not damaged, measured lipase activity and protein-bound phenols were not affected by wilting. However, when crushed or freeze/thawed, lipase activity was decreased and protein-bound phenols increased after 4 h of wilting compared to fresh. The increase in protein-bound phenols was highest when the forage was freeze/thawed. The lipase activity in red clover after FT with 4 h wilting or crushing or

Table 2. FA Composition Expressed as Grams per 100 g of FAMEs and Lipolysis [Calculated As (Proportion of Total FA in MLs at 0 h of Incubation − Proportion of Total FA in MLs at 1, 2, 4, or 8 h of Incubation)/Proportion of Total FA in MLs at 0 h of Incubation] of ML during Incubation of Red Clover Fresh and after Wilting (4 or 24 h) with Different Treatments [No Damaging, Crushing (Crush), or FT] (n=3)[a]

treatment	wilting (h)	incubation time (h)	C16:0	C18:0	C18:1 n-9	C18:2 n-6	C18:3 n-3	lipolysis
fresh	0	0	12.4	2.33	2.94	19.0	58.2	
		1	11.5	2.01	2.35	18.4	60.2	29.3
		2	12.3	2.18	3.15	19.3	57.7	33.4
		4	12.2	2.02	2.45	19.5	58.2	54.0
		8	12.9	2.10	2.82	20.7	55.9	61.6
no damage	4	0	11.7	2.14	2.52	17.0	60.9	
		1	11.8	1.98	2.14	16.8	61.3	25.9
		2	12.5	2.32	2.57	18.0	58.6	34.8
		4	11.8	1.91	1.99	17.2	61.1	50.0
		8	13.7	3.88	3.31	19.9	53.0	58.1
	24	0	14.4	2.18	2.50	17.1	57.4	
		1	14.1	2.06	1.80	16.8	58.5	24.2
		2	14.5	2.21	2.17	17.9	56.6	45.1
		4	15.2	2.30	2.18	18.4	54.9	57.5
		8	15.0	2.16	1.87	18.4	55.9	68.6
crush	4	0	12.4	2.11	2.12	16.3	60.2	
		1	12.6	2.10	1.79	17.1	59.5	22.9
		2	12.4	1.98	3.08	15.6	60.4	39.8
		4	12.7	2.05	1.89	17.0	60.3	49.8
		8	13.4	1.99	1.86	17.9	58.6	62.5
	24	0	14.3	2.19	2.46	18.2	55.9	
		1	14.6	2.14	2.50	18.3	55.3	27.4
		2	14.7	2.30	2.55	18.1	55.1	41.2
		4	15.0	2.24	2.54	18.9	54.3	42.5
		8	15.3	2.23	2.43	19.7	53.5	52.9
FT	4	0	12.7	2.06	2.05	16.6	59.4	
		1	13.0	1.98	2.12	16.9	58.6	29.3
		2	13.1	2.07	2.26	17.0	58.1	44.9
		4	13.3	2.01	2.16	17.1	58.5	52.1
		8	13.8	2.11	2.08	18.1	56.7	64.0
	24	0	15.8	2.47	2.80	16.5	53.9	
		1	16.5	2.61	3.02	16.3	52.3	7.43
		2	16.4	2.41	2.99	17.0	52.4	27.7
		4	16.3	2.44	3.00	16.9	52.7	37.3
		8	17.0	2.56	3.02	17.2	51.0	32.6
				Statistics				
treatment			***	NS	NS	*	***	***
wilting			***	NS	T	T	***	***
incubation			***	NS	NS	***	T	***
treatment × wilting			***	*	**	**	**	***
treatment × incubation			NS	NS	NS	NS	NS	*
incubation × wilting			NS	NS	NS	NS	NS	T
three-way-interaction			NS	NS	NS	NS	NS	**

[a] T = trend at $0.10 > p \geq 0.05$. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. NS = not significant ($p \geq 0.10$).

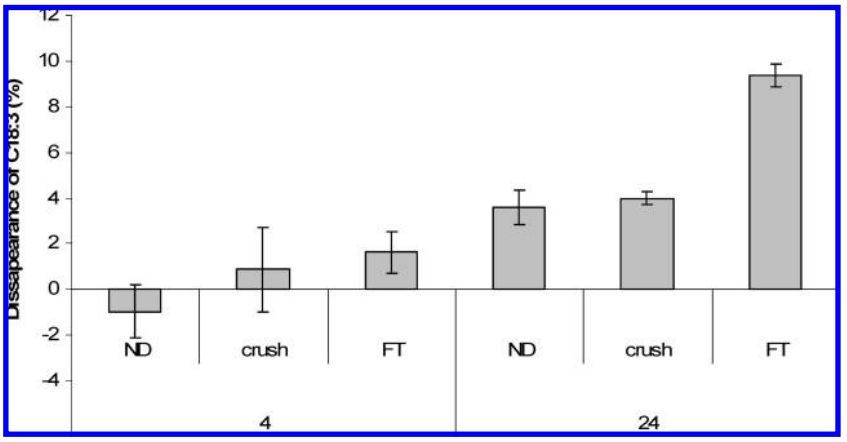

Figure 2. Disappearance of C18:3 after wilting (4 or 24 h) of treated [not damaged (ND), crushed, or FT] red clover calculated as (g of C18:3 per 100 g of fatty acid methyl esters (FAMEs) in fresh) − (g of C18:3 per 100 g of FAME in treated samples)/(g of C18:3 per 100 g of FAME in fresh). The error bars indicate the standard error of the mean (n = 3).

FT with 24 h wilting did not differ. Nonetheless, protein-bound phenols of these treatments did differ from the control.

The second part of experiment 2 considered the incubation of the ground red clover with and without damaging and differing wilting times, in a phosphate buffer with ascorbic acid. FA composition and lipid fractions after incubation for 0, 1, 2, 4, and 8 h were determined, and lipolysis was calculated. An overview of the results on FA composition is shown in **Table 2**. This illustrates a decrease in C18:3 and a concomitant increase in C16:0 during wilting of forages but also during incubation. This indicates FA oxidation, which is a degradation of unsaturated FAs. Furthermore, **Table 2** shows the rate of lipolysis, which is an indication of the degradation of the ML. Before incubation, the proportion of C16:0 was higher and the proportion of C18:3 was lower when the forage was damaged compared to undamaged. The wilting time had a similar general effect, but this was more pronounced when the forage was damaged, as indicated by the significant interaction between treatment and wilting time. The effect of treatment and incubation on other FAs was rather limited. Lipolysis of ML during incubation was extensive because up to 68.6% of the ML was hydrolyzed after 8 h of incubation. Nevertheless, lipolysis was lower in crushed red clover wilted for 24 h and lowest in FT red clover wilted for 24 h compared to all other treatments. In **Figure 2** the decrease in C18:3 during wilting and expressed as a proportion of FA methyl esters is presented. No decrease in C18:3 was observed in forages wilted for 4 h, regardless of damaging. In forages wilted for 24 h, however, a decreased proportion of C18:3 was found for all types of damaging but the decrease was highest in the FT forage.

In **Figure 3**, the proportion of the total FA over the three lipid fractions, ML, FFA, and neutral fraction, are presented. After wilting, ML was the largest fraction, containing up to 70 g per 100 g of total FA. When wilted for 4 h, there were no differences between the treatments for any of the fractions. However, when wilted for 24 h, 58.4 and 42.6 g 100 g^{-1} of total FA occurred in the ML fractions and 30.9 and 40.1 g 100 g^{-1} of total FA in the neutral fraction in the crushed and FT, respectively, whereas in the fresh forage, this was 67.7 and 22.7 g 100 g^{-1} of total FA in ML and neutral fraction, respectively. The proportion of total FA in the FFA fraction after 24 h of wilting was highest in the FT forages; the other treatments did not differ.

In the forages wilted for 4 h, no differences in lipid metabolism between treatments could be found during the incubation for all fractions. After 24 h of wilting, however, the proportion of total FA in the ML fractions was lowest and in the FFA fraction was highest in the not damaged forage after 8 h of incubation. Differences occurring between treatments in the neutral fraction after 24 h of wilting decreased during the incubation, to disappear after 8 h.

DISCUSSION

Experiment 1. The inhibition of enzymes as a result of bound phenols has been studied before (*14*−*16*). These studies showed that the binding of quinones, originating from phenols by non-enzymatic oxidation, is capable of binding to enzymes and, as such, inhibiting them. In particular, Rohn et al. (*16*) found a pH-dependent decrease (87.6−61.3%) in α-chymotypsin activity when chlorogenic acid, a phenol, was added to the incubation media. They concluded that this was due to covalent binding of phenols to the enzyme during the incubation. The enzyme lost activity as a result of lower affinity for the substrate, indicating that it was deformed and the substrate binding site was blocked as a result of binding of quinones. A higher amount of protein-bound phenols corresponding with a lower lipase activity, as indicated by our results, is in accordance with these results. This suggests that the lipolysis inhibiting role of PPO could be at least partially due to a direct inhibition of lipases through their binding with quinones, although a protection of the lipids, as a result of direct binding of quinones with the lipids or binding of quinones with proteins and thus encapsulating the lipids, cannot be excluded. When NaCN was added as an inhibitor of PPO, the amount of bound phenols occurring during the incubation with 4-methyl-catechol was lower. This indicates that PPO activity was linked with the increase in protein-bound phenols and, thus, with the inhibition of the measured lipase activity. Sodium cyanide has been described before as a noncompetitive PPO inhibitor in other plant species (*17*−*20*).

Experiment 2. An increase in the amount of protein-bound phenols over time can be a measure for the duration and the intensity of the PPO activity, whereas the measured PPO activity in a protein extract is rather an indication of the potential PPO activity present in a plant at a certain time point. The lack of a difference in protein-bound phenols between the fresh and undamaged forages after 4 h of wilting (**Figure 2**) showed that PPO was not active or only to a low extent. When the forage was crushed, 4 h was sufficient to generate an increase in protein-bound phenols from 0.75 to 4.77 nmol of tyrosine equiv (mg of protein)$^{-1}$ (**Figure 2**). This indicates that damaging can activate PPO, which has been suggested to be present in a latent form (*7*), in the fresh forage. They state that activation of red clover PPO can occur in two ways: by an interaction between the (oxidized)

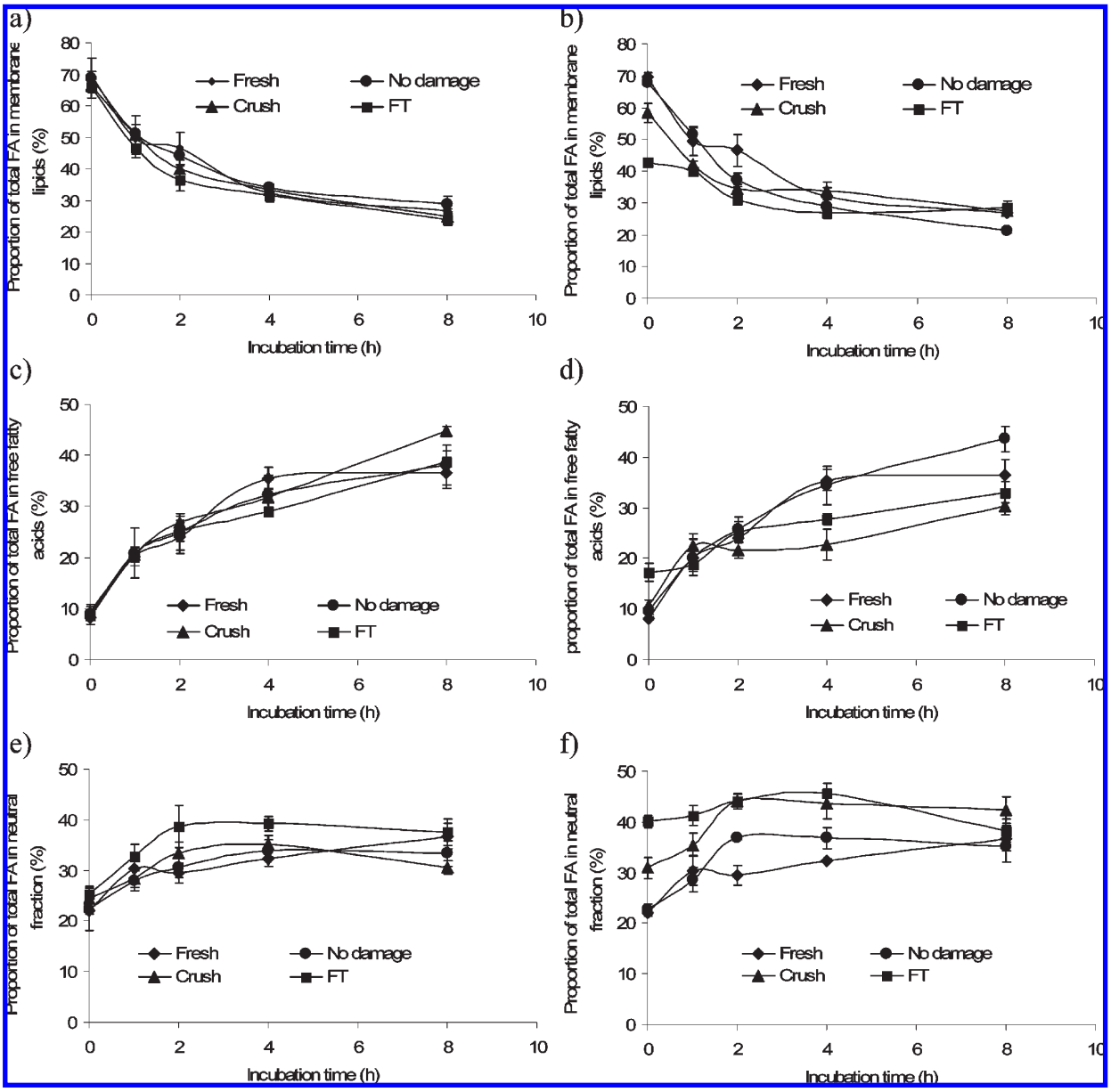

Figure 3. Changes in the proportion of total FAs in lipid fractions (ML, FFA, and neutral fraction) during incubation in a phosphate buffer with ascorbic acid of fresh, damaged (not damaged, crushed, and FT), or wilted [4 h (a, c, and e) or 24 h (b, d, and f)] red clover. The error bars indicate the standard error of the mean (n = 3).

substrate and the enzyme or by proteolytic cleavage of the latent PPO as a result of proteases. The first is a fast process because it can already occur, for instance, during the extraction of PPO. The latter is a rather slow process (*21*). Lee et al. (*7*) indicated that, after damaging, PPO activation is mediated by oxidized substrates of PPO. Because endogenous phenolic substrates are localized in vacuoles and PPO in the chloroplasts, PPO activition will only occur following disruption of cellular compartmentation. This would implicate that, in our FT treatment, PPO was most likely activated by the fast process of oxidized substrates. Similar to the first experiment, measured lipase activity increased when the amount of protein-bound phenols decreased. However, in the damaged forages, as opposed to the incubation of protein extracts with 4-methyl-catechol in experiment 1, there was not always a concomitant decrease in lipase activity when protein-bound phenols increased. This could indicate that not all extracted lipases are reachable for the quinones when in a cell structure. Therefore, the quinones bind to non-enzymatic proteins or to previously inhibited enzymes, resulting in an increase in protein-bound phenols without a decrease in measured lipase activity. However, it should be stated that the method used to determine protein-bound phenols will also determine non-protein-bound phenol complexes if these are insoluble in an 800 mL acetone L^{-1} solution, because this is used to precipitate the proteins. Although Sripad et al. (*22*) reported that the solubility of polyphenols in sunflower seeds in a 800 mL acetone L^{-1} water was low, precipitation of traces of polyphenols cannot be excluded. Because non-protein-bound phenol complexes (i.e., polyphenols) could occur as a result of polymerization of quinones with phenols, this could lead to an overestimation of protein-bound phenols and, thus, also explain the increase in protein-bound phenols without a decrease in measured lipase activity.

When the forages were incubated, the FA composition and lipid metabolism could be studied in more detail. Generally, the FA composition of the forages was within the line of the expectations (*23*−*25*). After wilting, a lower proportion of C18:3

(the most important unsaturated FA) and a higher proportion of C16:0 (the most important saturated FA) were found after 24 h of wilting compared to fresh. This indicates that oxidation of FA occurred when the forage was wilted for 24 h. The effect of wilting and wilting duration on oxidation in forages has been observed and studied before (*6*, *24*, *26*). However, the effect of damaging on oxidation of C18:3 in forages during wilting has not been reported. Our results suggest that more severely damaged forage FAs are more prone to oxidation. This would be explained by the stress-induced properties of lipoxygenases, enzymes responsible for the oxidation of FA and, in particular, C18:3 (*27*).

Because of the pretreatment of our forages (damaging and wilting), some processes already occurred before the incubation. This can be concluded from the differences in lipid fractions between treatments after wilting. Freeze/thawing and to a lower extent crushing seemed to have caused an increase in the neutral and FFA fraction during wilting for 24 h. The reason for this might be that lipases as well as diacylglycerol acyltransferases (DGATs) are stress-induced enzymes (*28*, *29*). Increases in TAG in forages during thawing (*30*) but also during wilting and ensiling (*6*), which were probably due to DGAT activity, have been reported earlier. The relatively high proportion of total FA in the neutral fraction, even in the fresh forage, could be due to thawing of the samples before extraction was started, because samples were stored frozen at −80 °C before incubation. During the incubation, however, the increase in FFA and neutral fraction in forages damaged and wilted for 24 h was lower, causing a lower proportion of FFA after 8 h of incubation compared to undamaged forages. In the neutral fraction, no differences between treatments occurred after 8 h of incubation. This shows that lipase and DGAT activity was lower in incubations of damaged forages compared to fresh, indicating an enzyme inhibition, possibly as a result of PPO activity. Lee et al. (*4*) studied lipid metabolism in red clover extracts during incubation in a buffer without ascorbic acid. They found increases in FFA, DAG, and to some extent in TAG and a decrease in ML. This is in accordance with the results from our incubations. In this study, cultivars with a higher PPO activity showed a lower increase in FFA and lower decreases in ML. The lack of ascorbic acid in their incubation buffer allows PPO activity during the incubation. In our buffer, ascorbic acid was added, implying that PPO activity could only occur before the incubation (i.e., during wilting). When PPO was activated by damaging and wilting for 24 h, the increase in FFA and decrease in ML were also lower compared to fresh forage when incubated. This confirms the role of PPO and indicates the importance of PPO activation in altering the plant lipid metabolism. Nonetheless, enzyme degradation (among which lipases) as a result of protease activity during wilting or incubation cannot be excluded because proteases will also be activated after damaging. However, the decrease in lipase activity observed in the current experiments are considerably higher than can be expected from protease-mediated enzyme degradation. After 4 h of wilting, the effect of damaging on lipid metabolism during incubation was limited, implying a minimum amount of time after damaging was required to induce differences in lipid metabolism. The discrepancy between the increase in the amount of protein-bound phenols and decrease in measured lipase activity in the damaged forages after 4 h of wilting on the one hand and the lack of difference in lipid metabolism during the incubation of the same forages on the other is hard to explain. It could be due to compartimentation in the plant cell, meaning that enzymes responsible for the lipid metabolism and PPO are in different locations in a plant cell or that a high degree of enzyme inactivation is needed before an effect on *in vitro* lipolysis can be seen. It certainly shows that there is not always a relation between measured lipase activity, which assesses the potential lipase activity and lipolysis of lipids during incubation in a buffer.

ACKNOWLEDGMENT

The authors highly appreciate the technical assistance of the staff of LANUPRO (Ghent University) and, in particular, An Ovyn and Sjarai Deschildre.

LITERATURE CITED

(1) Mayer, A. M. Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochemistry* **2006**, *67*, 2318–2331.
(2) Jones, B. A.; Muck, R. E.; Hatfield, R. D. Red-clover extracts inhibit legume proteolysis. *J. Sci. Food Agric.* **1995**, *67*, 329–333.
(3) Lee, M. R. F.; Scott, M. B.; Tweed, J. K. S.; Minchin, F. R.; Davies, D. R. Effects of polyphenol oxidase on lipolysis and proteolysis of red clover silage with and without a silage inoculant (*Lactobacillus plantarum* L54). *Anim. Feed Sci. Technol.* **2008**, *144*, 125–136.
(4) Lee, M. R. F.; Winters, A. L.; Scollan, N. D.; Dewhurst, R. J.; Theodorou, M. K.; Minchin, F. R. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. *J. Sci. Food Agric.* **2004**, *84*, 1639–1645.
(5) Sullivan, M. L.; Hatfield, R. D. Polyphenol oxidase and *o*-diphenols inhibit postharvest proteolysis in red clover and alfalfa. *Crop Sci.* **2006**, *46*, 662–670.
(6) Van Ranst, G.; Fievez, V.; De Riek, J.; Van Bockstaele, E. Influence of ensiling forages at different dry matters and silage additives on lipid metabolism and fatty acid composition. *Anim. Feed Sci. Technol.* **2009**, *150*, 62–74.
(7) Lee, M. R. F.; Tweed, J. K. S.; Minchin, F. R.; Winters, A. L. Red clover polyphenol oxidase: Activation, activity and efficacy under grazing. *Anim. Feed Sci. Technol.* **2009**, *149*, 250–264.
(8) Winters, A. L.; Minchin, F. R. Modification of the Lowry assay to measure proteins and phenols in covalently bound complexes. *Anal. Biochem.* **2005**, *346*, 43–48.
(9) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(10) Robert, C. M.; Cadet, F. R.; Rouch, C. C.; Pabion, M.; Richardforget, F. Kinetic study of the irreversible thermal deactivation of palmito (*Acanthophoenix rubra*) polyphenol oxidase and effect of pH. *J. Agric. Food Chem.* **1995**, *43*, 1143–1150.
(11) Lourenço, M.; Van Ranst, G.; Fievez, V. Difference in extent of lipolysis in red or white clover and ryegrass silages in relation to polyphenol oxidase activity. *Commun. Agric. Appl. Biol. Sci.* **2005**, *70*, 169–172.
(12) Van Ranst, G.; Fievez, V.; Vandewalle, M.; De Riek, J.; Van Bockstaele, E. Influence of herbage species, cultivar and cutting date on fatty acid composition of herbage and lipid metabolism during ensiling. *Grass Forage Sci.* **2009**, *64*, 196–207.
(13) Raes, K.; De Smet, S.; Demeyer, D. Effect of double-muscling in Belgian Blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids. *Anim. Sci.* **2001**, *73*, 253–260.
(14) Kroll, N. G.; Rawel, H. M.; Rohn, S. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003**, *9*, 205–218.
(15) Rohn, S.; Rawel, H. M.; Kroll, J. Inhibitory effects of plant phenols on the activity of selected enzymes. *J. Agric. Food Chem.* **2002**, *50*, 3566–3571.
(16) Rohn, S.; Rawel, H. M.; Wollenberger, U.; Kroll, J. Enzyme activity of α-chymotrypsin after derivatization with phenolic compounds. *Nahrung* **2003**, *47*, 325–329.
(17) Gulcin, I.; Kufrevioglu, O. I.; Oktay, M. Purification and characterization of polyphenol oxidase from nettle (*Urtica dioica* L.) and inhibitory effects of some chemicals on enzyme activity. *J. Enzyme Inhib. Med. Chem.* **2005**, *20*, 297–302.
(18) Yang, C. P.; Fujita, S.; Kohno, K.; Kusubayashi, A.; Ashrafuzzaman; Hayashi, N. , Partial purification and characterization of polyphenol oxidase fron banana (*Musa sapientum* L.) peel. *J. Agric. Food Chem.* **2001**, *49*, 1446–1449.

(19) Yilmaz, H.; Sakiroglu, H.; Kufrevioglu, I. Polyphenol oxidase from Mazruma grape (*Vitis vinifera* L.). *Agrochimica* **2003**, *47*, 21–27.
(20) Fujita, S.; bin Saari, N.; Maegawa, M.; Samura, N.; Hayashi, N.; Tono, T. Isolation and characterization of two phloroglucinol oxidases from cabbage (*Brassica oleracea* L). *J. Agric. Food Chem.* **1997**, *45*, 59–63.
(21) Winters, A. L.; Minchin, F. R.; Michaelson-Yeates, T. P. T.; Lee, M. R. F.; Morris, P. Latent and active Polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. *J. Agric. Food Chem.* **2008**, *56*, 2817–2824.
(22) Sripad, G.; Prakash, V.; Rao, M. S. N. Extractability of polyphenols of sunflower seed in various solvents. *J. Biosci.* **1982**, *4*, 145–152.
(23) Bauchart, D.; Verite, R.; Remond, B. Long-chain fatty-acid digestion in lactating cows fed fresh grass from spring to autumn. *Can. J. Anim. Sci.* **1984**, *64*, 330–331.
(24) Boufaied, H.; Chouinard, P. Y.; Tremblay, G. F.; Petit, H. V.; Michaud, R.; Belanger, G. Fatty acids in forages. I. Factors affecting concentrations. *Can. J. Anim. Sci.* **2003**, *83*, 501–511.
(25) Hawke, J. C. Chemistry and biochemistry in herbage. In *Lipids*; Butler, G. W., Bailey, R. W., Eds.; Academic Press: London, U.K., 1973; pp 213−263.
(26) Dewhurst, R. J.; King, P. J. Effects of extended wilting, shading and chemical additives on the fatty acids in laboratory grass silages. *Grass Forage Sci.* **1998**, *53*, 219–224.
(27) Feussner, I.; Wasternack, C. The lipoxygenase pathway. *Annu. Rev. Plant Biol.* **2002**, *53*, 275–297.
(28) Kaup, M. T.; Froese, C. D.; Thompson, J. E. A role for diacylglycerol acyltransferase during leaf senescence. *Plant Physiol.* **2002**, *129*, 1616–1626.
(29) Shah, J. Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. *Annu. Rev. Phytopathol.* **2005**, *43*, 229–260.
(30) Fievez, V.; Ensberg, M.; Chow, T. T.; Demeyer, D. Effects of freezing and drying grass products prior to fatty acid extraction on grass fatty acid and lipid class composition—A technical note. *Commun. Appl. Biol. Sci.* **2004**, *69*, 93–102.

Received March 13, 2009. Revised manuscript received May 29, 2009. Accepted June 09, 2009. The Ph.D. Grant of G. Van Ranst is funded by the Institute for Agriculture and Fisheries Research (ILVO), Belgium. Research is partially funded by IWT, Institute for the Promotion of Innovation by Science and Technology in Flanders, Belgium.