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Protection of polyunsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover

F. Gadeyne [a,1], G. Van Ranst [a,1], B. Vlaeminck [a], E. Vossen [a], P. Van der Meeren [b], V. Fievez [a,*]

[a] Laboratory for Animal Nutrition and Animal Product Quality, Faculty of Bioscience Engineering, Ghent University, Proefhoevestraat 10, 9090 Melle, Belgium
[b] Particle and Interfacial Technology Group, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

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ABSTRACT

Polyunsaturated fatty acid (PUFA) are to a large extent subject to biohydrogenation in a ruminal environment, which results to the healthy value of these PUFA being lost upon dietary addition to ruminants. PUFA are also prone to lipid oxidation upon storage. Therefore, it was tested whether emulsions could be protected against in vitro ruminal biohydrogenation and oxidation during storage by using protein extracts rich in polyphenol oxidase, an enzyme responsible for browning of plant tissues. PUFA rich emulsions were made with a protein extract from red clover (*Trifolium pratense* L.) before adding a synthetic diphenol (4-methylcatechol) to induce protection. Results after in vitro incubation confirmed the hypothesis and indicated the potential to prevent PUFA in linseed or fish oil from ruminal biohydrogenation and oxidation during storage through addition of 4-methylcatechol to the emulsions. Protection depended on the amount of oil present and protein concentrations in the emulsions. Protection efficiency increased with increasing the amounts of diphenol present in the emulsion per unit interfacial surface area. It is suggested that protection is caused by an effective encapsulation by cross-linking of the protein layer at the emulsion interface. For the first time, a method is described to protect PUFA using an enzyme abundantly available in nature, polyphenol oxidase, in combination with 4-methylcatechol.

1. Introduction

Consumers in the Western world are increasingly aware of healthy food. One strategy to achieve healthier animal products is to increase the content of polyunsaturated fatty acids (PUFA) in milk and meat and decrease the proportion of saturated fatty acids (Givens, 2008). This is possible by administration of PUFA rich oils to the diet of the animals (Dewhurst, Shingfield, Lee, & Scollan, 2006; Raes, De Smet, & Demeyer, 2004). PUFA are not only of interest for human health, *n-3* PUFA are also linked with better reproductive performances of animals (Gulliver, Friend, King, & Clayton, 2012). Increasing *n-3* PUFA levels in cattle diets may provide new alternatives for the development of nutritional-reproductive management strategies to improve reproductive efficiency (Coyne, Kenny, & Waters, 2011). However, unsaturated fatty acids are to a large extent subject to ruminal biohydrogenation (BH), resulting in the formation of saturated fatty acids and accumulation of cis and trans isomers (Fievez, Vlaeminck, Jenkins, Enjalbert, & Doreau, 2007) so that the healthy value for both humans and animals of these PUFA rich oils administered to ruminants is lost. Therefore, it is necessary to protect these PUFA against microbial BH in order to achieve an adequate transfer to the duodenum of PUFA without conversion.

One of the most efficient technologies to protect oils against ruminal BH is embedding oil droplets in a protein matrix followed by a formaldehyde treatment (Scott, Cook, & Mills, 1971). Here, a complex is formed between formaldehyde and amino acids in the protein matrix, resulting in a protective shell. It has been shown that it is possible to increase α-linolenic acid, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) levels in milk fat with formaldehyde-treated emulsified oils rich in these fatty acids (Kitessa et al., 2004; Sterk, Vlaeminck, van Vuuren, Hendriks, & Dijkstra, 2012). However, formaldehyde is a noxious product and use of it in the European Union is subject to strict regulations

Abbreviations: PUFA, polyunsaturated fatty acids; BH, biohydrogenation; EPA, eicosapentanoic acid; DHA, docosahexanoic acid; PPO, polyphenol oxidase; 4-MC, 4-methylcatechol; 4-HR, 4-hexylresorcinol; SPME–GC/MS, Solid Phase Micro-Extraction–Gas Chromatography/Mass Spectroscopy; TBARS, thiobarbituric acid reactive substances; RC + CAS, red clover extract with extra casein; CAS, casein.

* Corresponding author. Tel.: +32 92649002; fax: +32 92649099.
E-mail address: Veerle.Fievez@UGent.be (V. Fievez).

[1] These authors contributed equally to this manuscript.

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(2011/391/EU). Alternative technologies to by-pass PUFA exist, like calcium soaps, amide formation or composite gels (Jenkins & Bridges, 2007), but are not as efficient.

A natural mechanism to achieve protection may be promising in this respect. Van Ranst, Lee, and Fievez (2011) suggested that lipids could be effectively protected against pre-ruminal and ruminal lipid metabolism by encapsulation in protein–phenol complexes by the action of polyphenol oxidase (PPO), an enzyme abundantly present in nature, including red clover (*Trifolium pratense* L.) (Mayer, 2006). PPO is a generic name for a group of enzymes capable to ortho-hydroxylate monophenols (cresolase activity) and oxidise *ortho*-diphenols (catecholase activity) using molecular oxygen. The resulting *ortho*-quinones are highly reactive compounds, which are able to polymerise with other phenols or quinones or to bind with nucleophilic groups in amino acids such as –SH and $–NH_2$ groups, resulting in the formation of melanin-like protein–phenol complexes (Yoruk & Marshall, 2003). Lipids in red clover might be protected against degradation because of this mechanism by encapsulation of the chloroplast in a protein-phenol capsule (Lee, Tweed, Cookson, & Sullivan, 2010; Van Ranst et al., 2011). However, red clover is not a popular fodder and has a low fat content. Therefore, it should be interesting to use this mechanism to protect external sources rich in PUFA (e.g. linseed or fish oil) against ruminal BH.

In addition, it is important that the protected PUFA oils are chemically stable during storage. Oxidative stability of PUFA might be achieved by the protein-phenol capsule, as shown for multi-layered emulsions by minimising interactions between the encapsulated lipids and the aqueous phase surrounding it (McClements, 2012).

Hence, the current set of experiments evaluated, for the first time, the concept of protecting PUFA against rumen BH with PPO from a red clover extract. Furthermore, factors determining the protection efficiency were addressed. To achieve potential protection, emulsions were prepared using protein extracts from red clover, which served as a source of both PPO as well as emulsifier. Afterwards, the efficacy of these red clover stabilized emulsions to protect external PUFA against ruminal BH and oxidation during storage was assessed both with and without the supplementation of a diphenol.

2. Materials and methods

2.1. Materials

Red clover (*T. pratense* L. cv. Lemmon) was used as plant source in all experiments. It was sown in May 2010 (location: 50°59′4″N/3°47′6″E) and harvested 10 cm above ground level on different occasions during the growing season (March till October) of 2010 and 2011. Red clover received three fertilizer applications (in March and after the first and second harvest of the year; 6 kg N/ha, 20 kg P_2O_5/ha, 140 kg K_2O/ha). At harvest, red clover was at the early blooming stage. Plant material was frozen at −80 °C immediately after harvest. The cultivar Lemmon was used, as this cultivar is known for its high PPO activity (Van Ranst, Fievez, Vandewalle, De Riek, & Van Bockstaele, 2009).

Crude linseed oil (40.2, 194, 169 and 586 mg C18:0, C18:1*n-9*, C18:2*n-6* and C18:3*n-3* respectively per g total fatty acids) and fish oil (68.3, 179, 92.8, 38.6, 99.0, 187 and 112 mg C14:0, C16:0, C16:1*n-7*, C18:0, C18:1*n-9*, C20:5*n-3* and C22:6*n-3* respectively per g total fatty acids) were delivered by Dumoulin (Kortrijk, Belgium) and Nuscience (Drongen, Belgium) respectively. Casein was added to some (see further) emulsions as casein acid hydrolysate (Sigma, Diegem, Belgium). 4-methylcatechol was also purchased from Sigma–Aldrich (Diegem, Belgium). All other chemicals were of analytical grade and were purchased from either Sigma–Aldrich (Diegem, Belgium), Merck (Darmstadt, Germany), Carl-Roth (Karlsruhe, Germany) or VWR (Heverlee, Belgium), unless stated otherwise.

2.2. Preparation and characterisation of protected fatty acid emulsions: general procedure

A three-step process was performed: first, proteins were extracted from red clover, second, a PUFA rich oil was emulsified with this protein extract and third, creation of protein–phenol complexes was induced by adding a synthetic diphenol. It was chosen to use PPO from an extract from plant origin instead of pure PPO, as purchased PPO is generally of fungal origin and protein concentrations would be too low to deliver enough surfactant to obtain stable emulsions. As the hypothesis for this work originated from former studies with red clover (summarised by Van Ranst et al. (2011)), the latter was used as a vegetal source of PPO. A summary of emulsion characteristics in the different experiments is given in Table 1. Adjustments to the general procedure in the various experiments are mentioned in the Section 3.

Proteins were extracted according to Van Ranst, Fievez, De Riek, & Van Bockstaele (2009) with adaptations. In short, 150 g of frozen red clover, 500 ml of 0.1 M sodium phosphate buffer with 30 mM ascorbic acid (pH = 7.0), 0.5 g Triton X-100 and 1 g polyvinylpolypirrolidone were mixed in a blender for 1 min. After filtration and centrifugation (10,000×*g*, 15 min, 4 °C), acetone was added to the supernatant until a concentration of 800 ml acetone per litre total volume and kept at −18 °C for 35 min. After centrifugation (5000×*g*, 5 min, 4 °C) the pellet was re-dissolved in 400 ml of a 10 mM sodium phosphate buffer (pH = 7.0) without ascorbic acid. This concentrated protein extract was used to measure PPO activity and protein content as described by Van Ranst, Fievez, De Riek, et al. (2009). To determine PPO activity, the absorbance (A) was measured at 400 nm 20, 30 and 40 s after addition of 4-methylcatechol (4-MC). PPO activity was expressed as ΔA/min/mg protein. Folin–Ciocalteu reagent was added to the protein extract with copper and NaK-tartrate and the absorption was measured at 750 nm to determine the protein content, which was expressed as g protein per litre extract. PPO activity and protein content analysis was done in duplicate.

The concentrated protein extract, containing PPO, was further used to emulsify linseed or fish oil. First, coarse emulsions were created with a high speed Ultraturrax (T25 Basic, Ika Werke, Staufen, Germany), containing 20 g linseed oil per litre red clover protein extract, unless stated otherwise. These emulsions were put into a microfluidizer (M110S, Microfluidics Corporation, Newton, Massachusetts, USA) and passed five times to create stable emulsions with small droplet sizes, unless stated otherwise, at a compressed air pressure of 1.8 MPa, which corresponds to a liquid pressure of 25 MPa. Applying pressures higher than 25 MPa resulted in rather unstable emulsions which easily started to cream after passing through the microfluidizer. During processing, emulsions were cooled by passing through a heat exchanger coil immersed into an ice-water bath. Particle size distributions were checked immediately after preparation with a Mastersizer S (Malvern Instruments, Malvern, UK) equipped with a 300RF lens. The automated sample dispersion unit MS-17 (Malvern Instruments, Malvern, UK) was used. Data were analysed with the polydisperse model. Hereby, the real refractive index of the oil was fixed at 1.5295, whereas the imaginary refractive index was assumed to be 0.1000. Droplet sizes were characterised in terms of volume-weighted mean diameter (d_{43}), Sauter surface-weighted mean diameter (d_{32}), median volume-weighted distribution value $D[v,0.5]$ and 90% percentile of the volume-weighted distribution $D[v,0.9]$ using the available software (Malvern Instruments,