



Biohydrogenation Pathways for Linoleic and Linolenic Acids by *Orpinomyces* Rumen Fungus

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ABSTRACT : The objective of this study was to identify biohydrogenation pathways for linoleic, linolenic, oleic and stearic acids by *Orpinomyces* species of rumen fungus during *in vitro* culture. Biohydrogenation of linoleic acid produced conjugated linoleic acid (*cis*-9, *trans*-11 C18:2), which was then converted to vaccenic acid (*trans*-11 C18:1) as the end product of biohydrogenation. Biohydrogenation of linolenic acid produced *cis*-9, *trans*-11, *cis*-15 C18:3 and *trans*-11, *cis*-15 C18:2 as intermediates and vaccenic acid as the end product of biohydrogenation. Oleic acid and stearic acid were not converted to any other fatty acid. It is concluded that pathways for biohydrogenation of linoleic and linolenic acids by *Orpinomyces* are the same as those for group A rumen bacteria. (**Key Words :** Rumen Fungi, *Orpinomyces*, Biohydrogenation, Fatty Acids)

INTRODUCTION

The pathway for rumen bacterial biohydrogenation of linoleic acid (LA) involves three steps. The first step, yielding *cis*-9, *trans*-11 conjugated linoleic acid (CLA), is catalyzed by isomerase. In the second step, CLA is hydrogenated to produce *trans*-11 octadecanoic acid (vaccenic acid; VA). The final step is hydrogenation of VA to produce stearic acid (SA). This pathway was confirmed by studies involving incubation of unsaturated fatty acids with rumen contents *in vivo* and *in vitro* (Noble et al., 1969; Dawson and Kemp, 1970; Choi and Song, 2005; Choi et al., 2006; Wang et al., 2006; Zhang et al., 2006), and by studies with isolated rumen bacteria using pure-culture techniques (Eyssen and Verhulst, 1984; Song and Wang, 2003; Kim et al., 2005; Wang et al., 2005). Biohydrogenation of linolenic acid (LNA) involves four steps: isomerisation, followed by progressive hydrogenation to VA and then to SA (Kemp and Lander, 1983; 1984).

Kemp and Lander (1984) classified rumen bacterial isolates into groups A and B, according to pattern of biohydrogenation. The end product of group A bacterial biohydrogenation is VA; the end product of group B

bacterial biohydrogenation is SA. Only group B bacteria are able to hydrogenate oleic acid (OA) and VA to SA. Hence, to complete biohydrogenation of LA to SA, both group A and B rumen bacteria are needed.

Until recently, rumen fungi were thought not to be capable of rumen biohydrogenation. Biohydrogenation of LA by rumen fungi does occur, however, although the rate of biohydrogenation is considerably slower in fungi than in bacteria (Nam and Garnsworthy, 2007). Culture of isolated rumen fungal species indicated that *Orpinomyces* (GenBank numbers AF170189-191) demonstrates greatest capacity for biohydrogenation in the rumen (Nam and Garnsworthy, 2007). The objectives of the study reported here were to confirm the pathway for biohydrogenation of LA by *Orpinomyces* and to investigate possible biohydrogenation of LNA, OA and SA by this rumen fungus. These objectives were achieved by comparing disappearance of added fatty acids (AFA), and appearance of other fatty acids, when added to pure cultures of *Orpinomyces*.

MATERIALS AND METHODS

Culture conditions and biohydrogenation tests

Fungal cultures were grown anaerobically under O₂-free CO₂ in 12 ml tubes at 39°C, as described by Joblin (1981). Pure cultures of rumen fungi were prepared by culturing individual colonies picked from mixed rumen fungi grown on agar and an *Orpinomyces* culture was selected by

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Table 1. Composition (per litre) of hay sloppy medium used for culture of *Orpinomyces rumen fungus*

Component	Hay sloppy medium
Solution A ¹ (ml)	165
Solution B ² (ml)	165
Rumen fluid ³ (ml)	170
Resazurin ⁴ (ml)	2
NaHCO ₃ (g)	5
Yeast extract (g)	2
Peptone (g)	2
Cys-HCl.H ₂ O (g)	0.2
Agar (g)	1
Hay ⁵ (g)	3
H ₂ O ⁶ (ml)	≈485

¹ Solution A contained (g/L deionised water): KH₂PO₄, 3; NaCl, 6; (NH₄)₂SO₄, 3; CaCl₂, 0.3; and MgSO₄, 0.3.

² Solution B contained 3 g K₂HPO₄ L⁻¹ deionised water.

³ Rumen fluid was filtered through 4 layers of cheese cloth, centrifuged for 30 min at 11,000 g, autoclaved at 125°C for 15 min, centrifuged again for 30 min at 11,000 g, gently bubbled with O₂ free-CO₂ gas, and stored at -80°C until used.

⁴ 0.1% resazurin solution.

⁵ Field-cured grass hay (mostly *Lolium perenne*, *Lolium multiflorum* and *Phleum pratense*); oven-dried, then ground using a blender for 5 min.

⁶ Ultrapure water with 0.2 micron filtration (Purite Ltd, Oxford, UK); sufficient to make up to 1 L.

microscopy and its identity confirmed by DNA-sequence analysis (GenBank numbers AF170189-191), as described by Nam and Garnsworthy (2007). The culture medium used throughout the study was Hay Sloppy (HS) medium (Table 1), which was based on Bauchop (1979) and was prepared by the method of Hungate (1969). The culture was maintained by transfer of 10% to fresh HS medium every three days.

Fatty acid solutions containing LA, LNA, OA or SA in ultrapure water (Purite Ltd, Oxford, UK) with 20% BSA (Sigma, Poole, UK) as an emulsifier were prepared by the method of Kim et al. (2000). For biohydrogenation tests, a 1ml aliquot of *Orpinomyces* culture solution was added to 9ml fresh HS medium and pre-incubated at 39°C for 24 h. Fatty acid solutions (0.1 ml) were then added anaerobically to tubes (final fatty acid concentration 700 µM); tubes without fatty acid solution served as controls. Fatty acid solutions were added also to blank tubes containing only HS medium to check for any changes that were not due to fungal activity. Tubes were then incubated for 1, 3, 6, 9, 12, 24, 48, and 72 h. All incubations were performed in triplicate. The biohydrogenation reaction was stopped by putting tubes into ice water and tube contents were stored at -80°C until analysis for fatty acid contents.

Fatty acids analysis

Fatty acids were extracted from culture fluid using the hexane-isopropanol method of Hara and Radin (1978). Methyl esters of fatty acids (FAME) were prepared by the method of Christie (1982), as modified by Chouinard et al.

(1999), and quantified by gas chromatography as described by Feng et al. (2004). FAME were quantified using a gas chromatograph (6890; Agilent Technologies, Stockport, UK) equipped with a flame-ionization detector (7673 FID), autosampler (7683), automatic injector (7683), split injection port and a 100-m fused silica capillary column (i.d., 0.25 mm) coated with 0.2 µm film of cyanopropylpolysiloxane (CP-SIL 88; Varian). Hydrogen was used as the carrier (2.1 ml/min) and fuel gas (32 ml/min). All gases were passed through 7-µm inline filters (Nupro Co., Willoughby, OH). Injector temperature was 225°C and detector temperature was 255°C. Injection volume was 2 µl, with a split ratio of 1:100. Column temperature was held at 70°C for 4 min post-injection, increased to 110°C (8°C/min), increased to 170°C (5°C/min), held at 170°C for 10 min, raised to 225°C (4°C/min), raised to 240°C (20°C/min), and held for 5 min. Total run time was 50.5 min. Heptadecanoic acid (Sigma, Poole, UK) was used as an internal standard. Peaks were identified using pure methyl ester standards (FIM-FAME-7 mixture; Matreya Inc., Pleasant Gap, PA, USA; and CLA standard, Sigma, Poole, UK).

Statistical analysis

Peak areas for individual fatty acids were expressed as percentages of total area of identified peaks corrected for recovery of internal standard. No adjustment was made for fatty acids contained in the rumen fluid portion of HS medium because these were constant for all incubations and contributed only 1% of total fatty acids in treatment cultures. Statistical analysis was performed with the Genstat 9 statistical package (The Lawes Agricultural Trust, Rothamstead, UK). Proportions of fatty acids were analysed by using logistic regression employing a generalized linear model with a binomial distribution and logit link function. Effects of AFA and time were examined, together with their interaction. For fatty acids that were intermediate products of biohydrogenation, only time effects were examined because intermediate fatty acids were specific to each AFA, as described in the Results section.

RESULTS

Effects of AFA and time on proportions of initial and final fatty acids were highly significant ($p < 0.001$). Rate of disappearance of initial fatty acids, was significantly affected by AFA when all AFA were compared (AFA, time interaction: $p < 0.05$), but was not different when LA and LNA, or SA and OA, were compared. Rate of appearance of the final fatty acid (VA), was significantly affected by AFA when all AFA were compared and when LA was compared with LNA (AFA, time interaction: $p < 0.05$), but was not different when SA was compared with OA. All intermediate

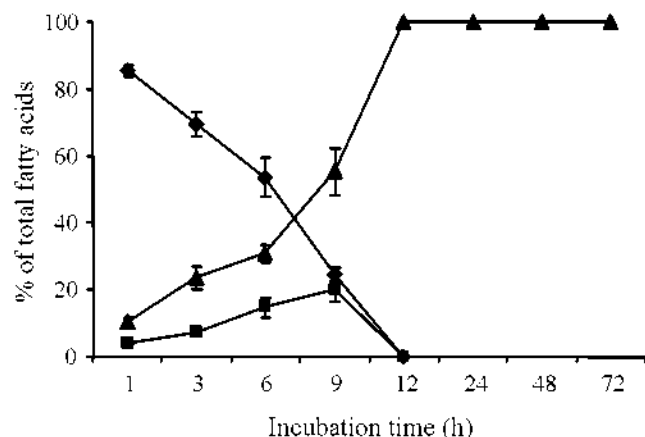


Figure 1. Biohydrogenation of linoleic acid by *Orpinomyces* rumen fungus. Points are means of triplicate determinations. ♦ linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid), ■ conjugated linoleic acid (*cis*-9 *trans*-11 octadecadienoic acid), ▲ vaccenic acid (*trans*-11 octadecenoic acid).

fatty acids changed significantly ($p < 0.001$) with time.

There was no change in fatty acid proportions in any control culture or blank tubes.

Biohydrogenation of linoleic acid

Biohydrogenation of LA by *Orpinomyces* was completely finished within 12 h incubation and LA proportion was only $24.6 \pm 2.1\%$ at 9 h incubation (Figure 1). The highest CLA proportion ($20.1 \pm 3.8\%$) was observed at 9 h incubation. VA proportion increased ($p < 0.001$) from the beginning of incubation, reached 100% at 12 h incubation and remained at 100% until the longest incubation time (72 h).

Biohydrogenation of linolenic acid

Biohydrogenation of LNA by *Orpinomyces* was completely finished within 24 h incubation. LNA proportion decreased at a rate similar to that of LA: LNA proportion was $22.9 \pm 3.7\%$ at 9 h incubation and no LNA was found at 12 h incubation. A conjugated triene (*cis*-9, *trans*-11, *cis*-15 C18:3) was detected after 1 h incubation and its maximum proportion was $18.6 \pm 3.9\%$ at 9 h incubation. A non-conjugated diene (*trans*-11, *cis*-15 C18:2) was also detected after 1 h incubation and its maximum proportion was $75.9 \pm 2.8\%$ at 12 h incubation. VA was detected only after 9 h incubation with LNA, which was significantly ($p < 0.001$) later than with LA. VA proportion increased to 100% at 24 h incubation, and remained at 100% until the longest incubation time (72 h).

Oleic and stearic acids

When *Orpinomyces* was cultured with OA or SA, proportion of each fatty acid remained at 100% throughout 72 h of incubation and no other fatty acid was detected.

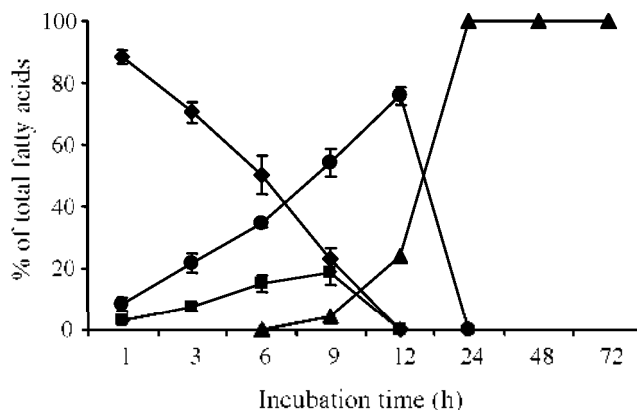


Figure 2. Biohydrogenation of linolenic acid by *Orpinomyces* rumen fungus. Points are means of triplicate determinations. ♦ linolenic acid (*cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid), ■ conjugated trienoic acid (*cis*-9, *trans*-11, *cis*-15 octadecatrienoic acid), ● non-conjugated dienoic acid (*trans*-11, *cis*-15 octadecadienoic acid), ▲ vaccenic acid (*trans*-11 octadecenoic acid).

DISCUSSION

Biohydrogenation of LA proceeded at a rate similar to that found in our previous studies of mixed rumen fungi and pure cultures of *Orpinomyces*, which was considerably slower than the rate of biohydrogenation observed for mixed rumen bacteria cultures (Nam and Garnsworthy, 2007). As in our previous studies, VA was the end product of LA biohydrogenation, with CLA as an intermediate. The absence of SA production from VA in these studies confirms that biohydrogenation of LA by *Orpinomyces* is the same as for Group A bacteria, which normally produce small amounts of CLA and high amounts of VA (Sachan and Davis, 1969; Yokoyama and Davis, 1971; Kemp et al., 1975; Song and Kennelly, 2003).

Biohydrogenation of LNA also proceeded only as far as VA. Due to the extra step in the biohydrogenation process, VA production from LNA was slower than for LA, although LNA disappeared at the same rate as LA. Group A bacteria mostly hydrogenate LNA to VA, via *cis*-9, *trans*-11, *cis*-15 C18:3 and *trans*-11, *cis*-15 C18:2, although some group A bacteria produce *trans*-11, *cis*-15 C18:2 as an end product from LNA, without the final conversion step to VA (Hazlewood et al., 1976). Production of these intermediates during biohydrogenation of LNA provides further evidence that the biohydrogenation process is the same for *Orpinomyces* as for Group A bacteria.

Group B bacteria can convert OA and VA to SA, but group A bacteria cannot (Kemp and Lander, 1984). When *Orpinomyces* was cultured with OA, no biohydrogenation activity occurred. SA can be converted to OA by Δ^9 -desaturase (Chillard et al., 2000), but there was no evidence for this conversion in the present study. These results are

the same as Group A bacterial biohydrogenation.

Body and Bauchop (1985) studied lipid composition of the rumen fungus *Neocallimastix frontalis* and reported 48% of total fatty acids were saturated and 52% were monounsaturated. Polyunsaturated fatty acids were not detected and the high proportion of monounsaturated fatty acids was explained by the obligate anaerobic lifestyle of *Neocallimastix frontalis*. Kemp et al. (1984) studied unsaturated fatty acid metabolism for the rumen fungus *Piromonas communis* by using ¹⁴C-labelled polyunsaturated fatty acids. They detected some *cis-trans* isomerization and Δ^9 -desaturase enzyme activity after 20-30 h incubation, together producing small amounts of CLA and VA, but they did not detect any SA. Therefore, our results indicate that, at least *in vitro*, fatty acid metabolism of *Orpinomyces* involves conjugated fatty acid production by *cis-trans* isomerization and VA production by reductase activity, but no Δ^9 -desaturase activity.

In the rumen environment, fungi are closely associated with cellulose-digesting bacteria. These bacteria benefit fungi by rapidly hydrogenating unsaturated fatty acids (Kepler et al., 1966; Kemp et al., 1975). Rapid biohydrogenation of fatty acids by bacteria masks the biohydrogenation capacity of rumen fungi in co-culture studies and *in vivo*, which is why fungal biohydrogenation has been demonstrated only recently (Nam and Garnsworthy, 2007). Independent biohydrogenation capacity would be beneficial for rumen fungi, however, in the micro-environment where they digest plant cells containing polyunsaturated fatty acids. The findings of the present study confirm those of our previous studies and demonstrate that *Orpinomyces* can also biohydrogenate LNA, which is the major fatty acid of forage plants.

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