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Microbially Safe Utilization of Non-Inactivated Oats (*Avena sativa* L.) for Production of Conjugated Linoleic Acid

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A microbially safe process for the enrichment of conjugated linoleic acid (CLA) in oats was developed. The process consists of hydrolysis of oat lipids by non-inactivated oat flour, followed by propionibacterium-catalyzed isomerization of the resulting free linoleic acid to CLA. The first stage was performed at water activity (a_w) 0.7, where hydrolysis of triacylglycerols progressed efficiently without growth of the indigenous microflora of flour. Thereafter, the flour was incubated as a 5% (w/v) aqueous, sterilized slurry with *Propionibacterium freudenreichii* ssp. *shermanii.* The amount of CLA produced in 20 h was 11.5 mg/g dry matter corresponding to 116 mg/g lipids or 0.57 mg/mL slurry. The oat flour had also the capability to hydrolyze exogenous oils at a_w 0.7. Sunflower oil, added to increase linoleic acid content in triacylglycerols 2.7-fold, was hydrolyzed rapidly. Isomerization of this oil-supplemented flour as a 5% slurry gave final CLA content of 22.3 mg/g dry matter after 50 h of fermentation, corresponding to 118 mg/g lipids or 1.14 mg/mL slurry. Storage stability of CLA in fermented oat slurries at 4 °C was good.

KEYWORDS: Conjugated linoleic acid; CLA; oats; *Avena sativa*; lipid hydrolysis; isomerization; linoleic acid; *Propionibacterium*

INTRODUCTION

The use of enzyme-active food matrixes as process aids is a tempting practice in food manufacturing. However, a problem associated with this approach is the lack of purification and heat inactivation of the enzyme-active food components allowing the indigenous microflora to remain viable. In addition, to gain the wanted enzyme action, the presence of excess water is most often required. These facts mean that non-inactivated food components are potential initiators for the development of microbiological hazards. Therefore, a challenge remains how to achieve a desired enzymatic reaction by a non-inactivated food component without provoking concomitantly microbial growth and possibly also toxin formation. These aspects are especially associated with the use of non-inactivated cereals.

The nutritionally beneficial properties of oats (*1*) make it an ideal material to be processed by its endogenous enzymes into products that will meet the requirements of modern consumer. An example of this has been the conversion of linoleic acid present in oat flour into health-beneficial conjugated linoleic acid (CLA) (*2*, *3*). In this method, the flour was used without inactivation to allow the endogenous lipase to produce free linoleic acid, which was subsequently isomerized by a food-grade micro-organism into CLA (*2*). To carry out the hydrolysis, the non-inactivated, lipase-active oat flours were incubated in excess water. However, under ambient conditions triacylglycerol hydrolysis in aqueous oat slurries requires several hours (*2*, *4*), which is sufficient for substantial microbial growth.

In intact dry oat kernels very little if any hydrolysis of lipids occurs (*5*, *6*), but crushing of the kernels initiates triacylglycerol hydrolysis, which continues at a slow rate for several months (*7*). This background knowledge aroused the question whether the hydrolysis of lipids in oat flour could be accelerated by adjusting the water activity (a_w) at a level, which is favorable for lipid hydrolysis but still unfavorable for the growth of indigenous micro-organisms. The present study describes the development of a process where a microbially safe lipid hydrolysis period is followed by an isomerization stage for the conversion of oat-based linoleic acid to CLA in oat slurries. The study also shows that the CLA content in the oat slurry can be further enhanced by supplementation of the oat flour with exogenous linoleic acid-rich oil.

MATERIALS AND METHODS

Oat Material and Lipid Hydrolysis. Dehulled, non-inactivated (nonheat treated) oat groats (*Avena sativa* L.), cultivar Belinda, used in the study were obtained from Finn Cereal Ltd. (Vantaa, Finland).

The groats were milled just prior to use. Water activity of milled flours was adjusted to 0.70 by spraying deionized water to flours. The relationship between moisture content and water activity of the nonsupplemented and sunflower oil supplemented oat flours was determined previously using saturated solutions of LiCl (a_w = 0.113), $MgCl_2$ (a_w = 0.328), K_2CO_3 (a_w = 0.432), $Mg(NO_3)_2$ (a_w = 0.529), and NaCl (a_w = 0.753). Final a_w values of the moistened flours were checked with a water activity meter (Testo AG, Lenzkirch, Germany).

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The moistened flours were sieved (500 μm) and stored in polyethene bags at 23 °C for 14 days. Samples for lipid hydrolysis analysis were taken at 1−4-day intervals and samples for microbiological analysis (11 g of flour in triplicate) at the beginning and end of the period. After the lipid hydrolysis period, the flours were stored at −80 °C. The lipid hydrolysis was studied also in 5% (w/v) oat flour slurries incubated for 17 h and in unmoistened flour stored for 28 days at 23 °C.

Supplementation of oat flour with sunflower seed oil (Sigma, St. Louis, MO) was performed by spaying the oil into moistened flour. The amount of oil added was 85 g per kg of dry flour. The linoleic acid content of the oil was 70%.

Microbial Strain and Culture Conditions. The bacterium used was *Propionibacterium freudenreichii* ssp. *shermanii* DSM 20270. The cells were cultivated at 30 °C for 48 h in lactate growth medium containing per liter 10 g of tryptone (LabM, Bury, England), 5 g of yeast extract (LabM), and 20 g of 50% sodium lactate solution (Merck, Darmstadt, Germany). The pH of the medium was adjusted to 7.0 before sterilization. The bacterial cells were harvested by centrifugation at 5900*g* for 15 min and resuspended in saline containing per liter 8.5 g of NaCl and 1 g of bacteriological peptone (LabM).

Isomerization Experiments. The hydrolyzed oat flours were suspended in water to yield slurries containing 5% (w/v) flour. The slurries were homogenized with an Ultra Turrax for 2 min at 24 000 rpm before use. Thermostabile α-amylase from *Bacillus amyloliquefaciens* (Fluka, Buchs, Switzerland) in 0.2 M phosphate buffer (pH 7.0) was added to the slurry at 1000 U per liter of slurry before autoclave sterilization for 15 min at 121 °C. One unit (U) of activity corresponds to the amount of enzyme, which liberates 1 μmol maltose per minute from soluble potato starch (Fluka) at pH 6.9 at 25 °C.

The isomerization reaction was performed in a Biostat MD 2 fermentor (B. Braun, Melsungen, Germany) at 30 °C. To 1.0 L of sterilized oat slurry, propionibacterial cell suspension in 35 mL of saline was added to yield a viable cell count of 1×10^{10} CFU/mL of slurry. Stirring applied was 150−200 rpm depending on the viscosity of the slurry. After 20 min, the pH of the slurry was elevated to pH 8.2−8.4 with 3 M NaOH and was automatically maintained at that level with 1 M NaOH during the fermentation. Samples for fatty acid analysis and viable counts were taken at appropriate intervals. Slurry samples for fatty acid analysis containing both the oat material and the bacterial cells were freeze dried. The fermented oat slurries enriched with CLA were stored at 4 °C and analyzed for CLA content after 30 days.

Analytical Methods. In fatty acid analysis, the fatty acids in oat flour samples or in freeze-dried oat slurry samples were subjected to the methylation procedure described by Suutari et al. (*8*). In this procedure, the fatty acids were saponified with 3.7 M NaOH in 49% methanol at 100 °C for 30 min and then methylated with 3.3 M HCl in 48% methanol at 80 °C for 10 min. For analysis of the isomeric composition of CLA, the methylation reagent used was 2.2 M HCl in 64% methanol. The methyl esters were extracted in hexane/methyl-*tert*-butyl ether solution (1:1), and the extract was washed with aqueous alkali. Analysis of the fatty acid methyl esters was performed by a Hewlett-Packard model 6890 gas chromatograph using an HP-FFAP column (25 m, 0.2 mm inside diameter, 0.33 μm film thickness) with a flame ionization detector. The column temperature was programmed from 70 to 200 °C at a rate of 25 °C/min. Nonadecanoic acid methyl ester (Sigma) was added to the samples as an internal standard.

For analysis of the amount and fatty acid composition of major lipid classes, the lipids were extracted from oat flour samples or freeze-dried oat slurry samples in dichloromethane/methanol (2:1) twice for 2 h. The extracts were combined and evaporated to dryness under nitrogen. The lipids were separated into the major classes by thin-layer chromatography (*4*). For oat flour samples, a mixture containing known amounts of triheptadecanoin (Sigma), dipentadecanoin (Sigma), heptadecanoic acid (Sigma), and dipentadecanoyl phosphatidylcholine (Sigma) was used as an internal standard. For oat slurry samples with the propionibacterium, nonadecanoic acid was substituted for heptadecanoic acid in the standard mixture. The separated lipid classes were scraped off from thin-layer plates and used for fatty acid analysis as described above. The degree of lipid hydrolysis was calculated as the proportion of free fatty acids from the sum of fatty acids in triacylglycerols and as free acids.

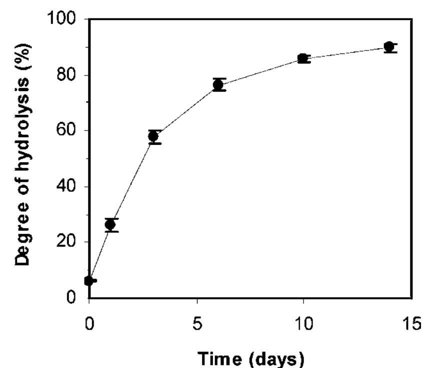

Figure 1. Progress of triacylglycerol hydrolysis in non-inactivated oat flour at a_w 0.70. The results are means ± SD ($n = 4$).

Viable counts of the propionibacterium were determined by the pour plate method on sodium lactate agar containing per liter 10 g of yeast extract (LabM), 5 g of tryptone (LabM), 10 g of β-glycerophosphate (Merck), 17 mL of 50% sodium lactate solution (Merck), and 12 g of agar (LabM). The plates were incubated anaerobically (Anaerocult A, Merck) at 30 °C for 6 days.

Total viable counts were established by the pour plate method on Plate count agar (LabM). The colonies were enumerated after incubation at 30 °C for 72 h.

Yeasts and molds were determined by plating on Potato dextrose agar (LabM). As a selective agent, chloramphenicol (Sigma) was used at a final concentration of 100 mg/L. The plates were incubated at 25 °C for 5−6 days.

Statistical Methods. The data represent the mean of four to six samples of two to three independent trials. The results are reported as the means and standard deviations (SD). The student's *t*-test was used to evaluate statistically significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Hydrolysis of Oat Lipids. The production of CLA by microbial isomerization requires the presence of linoleic acid as free acid. Therefore, the initial stage is to release linoleic acid from the lipids of ground oats. This raised the interest to test whether the hydrolytic reaction would progress fast enough and reach sufficient degree of hydrolysis when the water activity of the flour is adjusted to a level where the indigenous oat microflora is not expected to proliferate.

The oat flour used for these experiments contained total fatty acids 93 mg/g dry matter, of which the total amount of linoleic acid was 38 mg/g dry matter. From this linoleic acid 75% was bound in triacylglycerols. Fatty acids from the storage lipids were released by increasing the water activity of the flour from the storage level of a_w 0.20 (moisture content 5.5%) to a_w 0.70 (moisture content 12%), where most micro-organisms are unable to proliferate (*9*, *10*).

The hydrolysis of triacylglycerols progressed rapidly at a_w 0.70 (**Figure 1**). Hydrolysis of polar lipids was negligible. The amount of free linoleic acid was 19 mg/g dry matter at day three and after hydrolysis period of 14 days, the corresponding value was 30 mg/g.

For comparison, in aqueous suspension of non-inactivated oat flour (5% w/v, a_w about 1) the degree of lipid hydrolysis and the amount of free linoleic acid after 17 h were 41% and 10 mg/g dry matter, respectively. Prolonging of the incubation under these conditions would further increase the hydrolysis (*4*), but as shown later under the heading "Oat-Borne Microflora during Lipid Hydrolysis Period", growth of the indigenous microbes was substantial already after 17 h incubation.

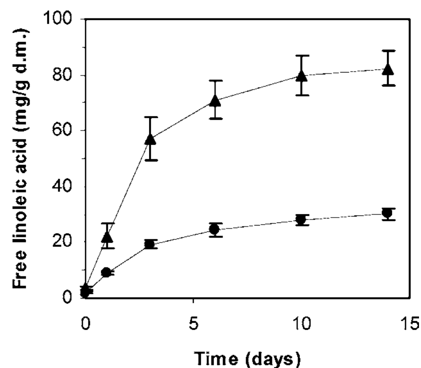

Figure 2. Formation of free linoleic acid in nonsupplemented oat flour (●) and in oat flour supplemented with sunflower oil (▲), each at a_w 0.70. Sunflower oil supplementation was 85 g per kg of dry flour. The results are means ± SD ($n = 4$).

In unmoistened (a_w 0.20) and non-inactivated oat flour, low water activity was clearly the rate-limiting factor for the hydrolysis. The degree of lipid hydrolysis was 34% 14 days after milling and 51% after 28 days of storage. Then, the amount of free linoleic acid had increased to 14 mg/g dry matter.

On the basis of these results, it can be concluded that water activity of 0.70 creates favorable conditions for hydrolysis of oat lipids.

Isomerization of Oat-Based Free Linoleic Acid to CLA. The high concentration of free linoleic acid released in oat flour at a_w 0.70 aroused further questions if the free linoleic acid formed in the flour matrix is available for and tolerated by the isomerizing propionibacterium. To test this, the hydrolyzed flour was mixed in excess water as 5% (w/v) oat slurry, sterilized, and inoculated with a culture of *Propionibacterium freudenreichii* ssp. *shermanii*. The pH of the isomerization was adjusted to 8.2−8.4, since alkaline pH has previously been shown to favor CLA formation in aqueous oat slurries (*2*).

Isomerization of free linoleic acid to CLA initiated without delay, indicating that at least a portion of the free linoleic acid released in the presence of limited water and thereafter sterilized in the presence of excess water was readily available for the cells. The highest rate of CLA formation, 1.5 mg/g dry matter per hour, occurred during the first 4 h of fermentation. The amount of CLA produced in 20 h was 11.5 mg/g dry matter corresponding to 116 mg/g lipids or 0.57 mg/mL slurry. The proportion of the *cis*-9,*trans*-11 isomer was 79% of the total CLA formed. During the fermentation, 40% of the initial free linoleic acid was converted to CLA. The remaining free linoleic acid may have been buried into the flour particles or in inclusion complexes formed and became so unavailable for the propionibacterium.

Hydrolysis of Lipids in Sunflower Oil-Supplemented Oat Flour. Since the storage lipids of oat flour were hydrolyzed rapidly at a_w 0.70, it was of interest to test whether the flour could be used for the hydrolysis of added linoleic acid-rich edible oil at the same a_w level. By supplementation of 85 g sunflower oil per kg of dry flour, the total linoleic acid content of the flour was increased to 88 mg/g dry matter, of which 87% was in triacylglycerols. Thus, the amount of linoleic acid in triacylglycerols was 2.7-fold in comparison with nonsupplemented oat flour.

During the lipid hydrolysis period at a_w 0.70, the amounts of fatty acids released were much higher than expected on the basis of the amount of oat lipids alone (**Figure 2**). In fact, the initial rate of hydrolysis in oil-supplemented flour was 2.8-fold compared with the nonsupplemented flour. These observations indicated that fatty acids were released from both exogenous and oat triacylglycerols. After the hydrolysis period of 14 days, the amount of free linoleic acid in sunflower oil-supplemented flour was 82 mg/g dry matter corresponding to 92% degree of hydrolysis. Thus, supplementation of oat flour with linoleic acid-rich oil offers an attractive means to increase the amount of free linoleic acid for CLA production in oat-based materials.

Table 1. Effect of Storage at $a_w = 0.70$ on Indigenous Microflora of Non-Inactivated Oat Flour and the Same Flour Supplemented with Sunflower Oil[a]

oat flour	total viable count		yeasts and molds	
	start	14 days	start	14 days
nonsupplemented	4.48 ± 0.13	4.28 ± 0.22	2.41 ± 0.27	2.40 ± 0.28
oil-supplemented	4.57 ± 0.22	4.23 ± 0.39	2.94 ± 0.57	2.77 ± 0.75
			(3.45 ± 0.06)[b]	(3.39 ± 0.31)[b]
			(2.43 ± 0.35)[b]	(2.14 ± 0.50)[b]

[a] The flours were stored at 23 °C for 14 days. Counts expressed as log CFU/g dry matter of mean values ± SD ($n = 6$). [b] Values in two separate trials with oil-supplemented flours ($n = 3$).

Noninactivated oats have also earlier been used to hydrolyze exogenous lipids. For example, Parmar and Hammond (*11*) hydrolyzed fats and oils utilizing the endogenous oat lipase. However, the oat caryopses used were moistened to 20% moisture content.

Oat-Borne Microflora during Lipid Hydrolysis Period. Seeds of cereal plants are known to harbor a large number of different types of micro-organisms (*12*). In the non-inactivated oat flours used in the present study, bacteria and low levels of yeasts and molds were detected. After moistening of the flours to a_w 0.70, possible growth of this indigenous microflora was followed for 14 days. According to **Table 1**, during this period the micro-organisms did not proliferate ($p > 0.05$), either in the nonsupplemented or in the oil-supplemented flour. This is in accordance with literature information that at a_w 0.70 growth of micro-organisms, except a few xerophilic molds, is inhibited (*9*, *10*). Further, mycotoxin production by toxigenic fungi requires a_w environments of $a_w \geq 0.80$ (*9*, *13*−*14*). As no microbial growth occurred during the lipid hydrolysis period in flour, loss of oat-based nutrients can also be suspected to be minimal.

For comparison, the lipid hydrolysis was performed also in aqueous 5% oat slurry (a_w about 1) prepared from non-inactivated oat flour. Then, the indigenous microflora of oat flour grew rapidly. The total viable counts increased during 17 h from log CFU/mL 3.52 ± 0.27 to 6.54 ± 0.20 ($n = 4$). Also the counts of fungi increased from log CFU/mL 2.38 ± 0.22 to 4.40 ± 0.20 ($n = 4$). These results emphasize the importance of controlling the water activity of non-inactivated flour to ensure microbial safety and feasibility e.g. for a subsequent CLA enrichment process.

Isomerization in Oat Slurries Containing Sunflower Oil. Flour supplemented with sunflower oil and hydrolyzed at a_w 0.70 similarly as nonsupplemented flour was suspended in excess water as 5% slurry, sterilized, and inoculated with propionibacterium. The time course of CLA formation was distinctly different from that observed with nonsupplemented flour (**Figure 3**). The formation of CLA initiated after a lag phase of at least 6 h. Between 20 and 28 h of fermentation, the rate of formation was 0.75 mg/g dry matter per hour. Final concentration of CLA was 22.3 mg/g dry matter after 50 h of fermentation corresponding to 118 mg/g lipids or 1.14 mg/mL slurry. Thus, when oil-supplemented flour with the 2.7-fold

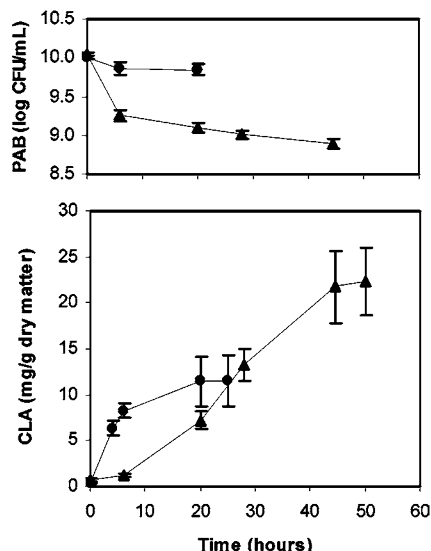

Figure 3. Production of CLA by *Propionibacterium freudenreichii* ssp. *shermanii* cells (PAB) in 5% (w/v) oat slurries prepared from nonsupplemented oat flour (●) or oat flour supplemented with sunflower oil (▲). Sunflower oil supplementation was 85 g per kg of dry flour. Prior to fermentation, linoleic acid was released from triacylglycerols by adjusting a_w of the flours to 0.70. Fermentations were performed at 30 °C, and the pH of slurries was maintained at 8.2–8.4. The results are means ± SD ($n = 6$).

increased content of free linoleic acid was used for isomerization, the amount of CLA formed was 1.9-fold higher than without supplementation. At the end of fermentation, 32% of the initial free linoleic acid had been consumed.

The isomerization of linoleic acid to CLA in oat slurries occurs without growth of the propionibacterium (*2*). In sunflower oil-supplemented slurries, the viable count of propionibacterium decreased rapidly to less than one-sixth of initial (**Figure 3**), probably due to the high concentration of free linoleic acid (*15*). Nevertheless, the formation of CLA, once initiated, continued with the remaining viable cells at least for 35 h reaching surprisingly high final levels. This time period of CLA formation was considerably longer than periods reported for efficient CLA production by nongrowing cells of *P. freudenreichii* ssp. *shermanii* in fed-batch cultivations in whey-based medium or in reaction systems where concentrated cell suspensions were incubated in a phosphate buffer solution in the presence of micellar free linoleic acid (*16*, *17*).

Storage Stability of CLA in Oat Slurries. It has been reported that concentrated preparations of CLA undergo rapid oxidative degradation when exposed to air at elevated temperatures (*18*−*20*). When the present CLA-enriched oat slurries were stored at 4 °C, only a slight if any decrease in CLA occurred during 30 days. The percentage of CLA remaining from original CLA was 87.8 ± 2.0 and 102.3 ± 7.0 ($n = 4$) in slurries from nonsupplemented and oil-supplemented oat flours, respectively. The oat matrix may have had a protective effect against the oxidative free radical chain reactions. On the other hand, as some of the propionibacterial cells stayed viable in the CLA-containing oat slurry, the possibility remains that a loss of preformed CLA is masked behind slow CLA formation during the storage period at 4 °C. In any case, the results favor the view that the content of CLA in chill-stored food materials stays relatively stable.

Previously, microbial production of CLA has been carried out in synthetic media or in dairy-based matrixes most often supplemented with free linoleic acid (*21*−*25*). The present results suggest that oat is a plausible candidate to be converted into an edible CLA concentrate. As a whole, the present study succeeded in combining different pieces of existing knowledge and developing them into a novel process. The single stages of the method or the components used are either natural or been in use for other food manufacturing purposes. The benefits over existing methods are obvious: oat lipids can be hydrolyzed without a risk of harmful microbial proliferation, the hydrolysis step can be done separately from the aqueous isomerization, and the overall handling of liquid is essentially reduced. In addition, the amounts of CLA produced are at least as high as reported previously. The method also enables supplementation of the oat flour with linoleic acid-rich oil without the risk of oil−water phase separation and so offers further possibilities to enhance the concentration of CLA.

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