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Citation	Journal of the science of food and agriculture, 102(10), 4355-4362 https://doi.org/10.1002/jsfa.11789
Issue Date	2022-08-15
Doc URL	https://hdl.handle.net/2115/90288
Type	journal article
File Information	JSFA_11789.pdf



**A study of lipolysis induced by adjuncts from edible *Aspergillus* sp.
solid culture products on ripened semi-hard cheese**

**Short running title: Lipolysis induced by edible *Aspergillus* sp. adjunct on ripened
cheese**

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ABSTRACT

BACKGROUND: *Aspergillus* sp. has been used in traditional Japanese fermented foods. The protease-containing culture products of *A. oryzae* have been applied as the adjunct enzyme source to enrich the flavor in ripened cheese. Although proteolysis was stimulated, the increase of free fatty acids (FFA) was recognized in some products. Since an excess amount of FFA accumulation could cause rancidity in cheese products, the assessment of lipase activity was considered to be essential for the cheese adjunct preparation.

RESULT: Although an equal lipase activity from the adjunct materials of *A. kawachii* NBRC 4308, *A. luchuensis* RIB 2604, and *A. oryzae* AHU 7139 was applied to semi-hard cheese, the FFA level was significantly higher in *A. oryzae* cheese than in the others. Furthermore, the profiles of volatile components were different in experimental cheeses. Application of *in vitro* study with experimental curds demonstrated that the high FFA might not depend on the lipase retainability on curds. On the contrary, the pronounced activation of the lipases occurred in *A. oryzae* after incubation with the curds. Moreover, incubation of the insoluble lipase that had been attached to the cells with skim milk curd extracts allowed the release of lipases from the cells into the medium with remarkable activation.

CONCLUSION: *A. oryzae* AHU 7139 possessed a complex lipolytic system comprising extracellular and cell-binding lipases that were attributed to the increase in FFA in *A. oryzae* cheese.

KEYWORDS: lipase; *Aspergillus* sp.; curd peptides; free fatty acids; ripened cheese

INTRODUCTION

The genus *Aspergillus* has been used in traditional Japanese fermented products such as miso, shoyu, and alcoholic beverages.^{1,2} In the traditional protocol, suitable *Aspergillus* strains have been seeded on solid cereal materials such as steamed rice, soybean, and wheat for koji preparation to obtain enzyme cocktails containing amylases and proteases, which contribute to the fermentation of the corresponding products.³ Genomic analysis of *A. oryzae*, a representative species of this genus, revealed that it encodes about 12,000 genes, 135 of which are thought to be related to proteases.⁴ Focusing on this high proteolytic potential along with its food safety, we have proposed a semi-hard cheesemaking protocol with the solid culture products of *A. oryzae* as flavor-enrichment adjuncts.⁵ In this protocol, an adjunct material was mixed with the fresh cheese curds followed by pressing and ripening to stimulate proteolysis in the products. Although *A. oryzae* adjuncts promoted proteolysis during cheese ripening, it led to a remarkable free fatty acid (FFA) accumulation in some cheese products. Although FFA accumulation during cheese ripening could be attributed to the action of lipases from several sources,⁶ involvement of endogenous milk lipase is unlikely when the cheese milk had been pasteurized due to its heat lability.⁷ Moreover, added rennet and starter culture activity had less impact of lipolysis than the additive *Aspergillus* culture products which was monitored in the control cheese.⁵ Thus, the lipase from an adjunct material was thought to be the significant factor for FFA accumulation. Pronounced release of volatile fatty acids from milk fat could cause an undesirable flavor defect in the finished cheese products known as rancidity.⁸ Accordingly, in order to take advantage of the proteolytic potential of *A. oryzae*, not only proteases but also lipases should be investigated when *Aspergillus* culture products are intended to use for cheesemaking adjuncts.

Based on the simple presumption, if the fungal enzyme activity levels in the adjuncts increase, their respective reaction products in cheese would be parallelly increased. In fact, Kataoka et al.⁹ used several commercial proteases to accelerate cheese ripening with the similar cheesemaking procedure as we did.⁵ They found that the water-soluble nitrogen (WSN), an index of cheese maturity, increased in a dose-dependent manner when they added the same enzyme source in different amounts. However, they recognized that the effect did not always correlate with the protease activity when different protease sources were used. Specifically, the WSN level of 4-month-old cheese using 0.05 U of acidic protease from *A. oryzae* was comparable to that of using 0.005 U of neutral protease from *B. subtilis*. Regarding lipase, similar inconsistency between lipase activity and FFA in the resulting products can be postulated.^{10,11} Considering the hydrophobic feature of cheese curds, formed by a network of paracasein micelles, hydrophobic interaction is likely to occur between cheese curds and the lipases during the mixing procedures of the adjuncts into curds followed by pressing with whey drainage. Assuming that the entrapment of the lipase molecules in the curds specifically depends on the degree of its affinity toward cheese curds, the correlation between lipase activity in a supplied adjunct and the resulting FFA content in ripened cheese products would be unpredictable.

In this study, we prepared three adjunct materials containing an equivalent lipase activity using different species of *Aspergillus* to mix with the fresh cheese curds followed by pressing and ripening in order to compare the FFA amount in the products. Since a remarkable increase of FFA in the cheese was recognized with the adjunct of *A. oryzae*, its cause was investigated from the viewpoint of the retainability of lipases on the curds with a simulated experiment to measure the soluble lipase activity before and after incubation with the curds. Furthermore, as the prepared adjunct material was

proved to include both extracellular and intracellular lipases¹², fractionation of the lipases from *A. oryzae* adjunct materials was carried out to specify the fraction responsible for the FFA increase in *A. oryzae* cheese. Finally, we proposed the possible mechanism of FFA accumulation during cheese ripening that would take place when the adjunct materials from *A. oryzae* are supplemented.

MATERIALS AND METHODS

Strain and culture condition

The strains used in this study were as follows; *A. kawachii* NBRC 4308 from Biological Resource Center (Chiba, Japan), *A. luchuensis* RIB 2604 kindly gifted by National Research Institute of Brewing (Hiroshima, Japan) and *A. oryzae* AHU 7139 from the culture collection of Hokkaido University. Then the spore suspension was prepared as described in previous study¹² to a concentration of 2.5×10^5 spores mL⁻¹.

Twenty-five grams of WPC80 (Fonterra, Auckland, New Zealand) was dissolved in 75 g of deionized water and adjusted to pH 4.0 with lactic acid. The solution was divided (10 g) into 100 mL Erlenmeyer flask each and autoclaved at 121°C for 15 min to prepare the solid medium. The medium was inoculated with 150 µL of the spore suspension and cultivated at 20°C for 7 days.

Preparation of adjunct materials for cheese making

The adjunct material from culture products (CP) was prepared by recovery of the 10 g CP from flasks, pooled to freeze dry and then ground with a mortar and pestle to obtain freeze-dried powder (FDP), which was regarded as the adjunct material. On average, 0.27 g of FDP was obtained from 1 g of CP. As a control, un-inoculated solid medium was prepared likewise to obtain un-inoculated freeze-dried powder (UIFDP).

Measurement of enzyme activity in the adjunct materials

The suspension of the FDP (0.27 g) dispersed in 2 mL of 0.02 mol L⁻¹ sodium phosphate buffer, pH 7.0 was used. The lipase activity was determined using 1 g of butter oil emulsified with 100 mL of 2% polyvinyl alcohol (degree of polymerization 2000, Kishida Chemical, Osaka, Japan) as the substrate. The substrate was divided into 2 mL into each test tube and subjected to pre-incubation at 15°C for 5 min, followed by an addition of the enzyme to be tested (50 µL) and 0.5 M sodium phosphate-citric acid buffer pH 5.5 (250 µL). The reaction was carried out at 15°C for 30 min and terminated by adding 7.5 mL of extract solution (heptane : isopropanol : 0.5 mol L⁻¹ sulfuric acid = 48:48:4). The extracted FFA was determined by the phenol-red method.^{13, 14} Enzyme activity was expressed as µmol of released oleic acid from the substrate per 1 h at the defined temperature from 1 g of the CPs (1 µmol oleic acid/h/g-CP = 1 lipase unit: LU).

The proteolytic activity was determined as previously described with some modifications.¹² The enzyme reaction was incubated at 30°C for 1 h. Enzyme activity was expressed as µmol of released tyrosine per h at 30°C extracted from 1 g of the CPs (1 µmol tyrosine/ h/ g-CP = 1 protease unit: PU).

Cheesemaking

The cheesemaking was carried out according to the conventional procedure for Gouda-type cheesemaking as previously described.¹² Raw milk was obtained from the experimental farm in the Field Science Center for Northern Biosphere, Hokkaido University, and 105 kg of standardized milk (3% fat) was used in this study. After whey drainage, 1 kg of the recovered curds was mixed with each FDP and UIFDP mixture (total 2.16 g) to adjust to an equivalent lipase activity in all prepared experimental cheeses, and only UIFDP was mixed with the curds as the control cheese (Table 1). The

control and experimental cheeses were prepared in duplicate. Following brief pressure (1.0 kg/cm² for 15 min), inversion and the second stage of pressure (1.2 kg/cm² for 50 min) were performed. The 1 kg cheese blocks were cooled in water overnight then salted with brine (25% NaCl, w/w) for 12 h. The cheeses were matured at 11.5°C with a relative humidity of 85% for 12 weeks. After 28 days of the manufacture, the cheese surface was coated with wax (Paramelt, Netherland) and left for ripening.

Chemical analysis of cheese

The moisture, fat, protein, and water soluble nitrogen (WSN) were determined as previously described.¹² For free fatty acids (FFAs) determination, the cheese sample (0.2 g) was transferred to 2 mL of 7.7 M HCl in a test tube and placed into boiling water to dissolve the sample. FFAs were extracted by the same extraction reagent as was used in the lipase activity measurement to determine by the phenol-red method¹⁴ and its content was expressed as (mmol) in 1 kg of cheese using oleic acid as the standard. Salt calculated from chloride content were measured as previously described.⁵ All assays were performed in triplicate.

Volatile compound analysis in cheese

The cheese samples were chopped by food processor and 5 g samples were weighed in 20 mL vials and closed with a PTFE septa and magnetic cap. Volatile organic compounds (VOCs) in the cheese were extracted using a headspace solid-phase micro-extraction (SPME) technique with an auto-sampler (AOC-5000 plus; Shimadzu Co., Kyoto, Japan). The vials were placed into a heat block at 45°C for 5 min, then absorbed into SPME fiber (2 mm of 50/30 µm DVB/CAR/PDMS fiber; Supelco, Bellefonte, PA) at 45°C for 20 min. Before analysis of the first sample, the fiber was baked at 250°C for 30 min. After absorption, the fiber was inserted into the gas chromatograph/mass

spectrometer (GCMS-QP2010 Ultra; Shimadzu Co., Kyoto, Japan). The analysis was conducted in split mode with the following conditions: injector temperature 250°C; split ratio 5:1; helium carrier gas; linear velocity 50 cm/s. The VOCs were separated on a fused silica capillary column (DB-WAX 30 m length × 0.25 mm internal diameter, Agilent Technologies Inc., Santa Clara, CA) with a temperature-rising condition (initial oven temperature at 40°C, held at 40°C for 1 min, increased by 16°C/min to 120°C, increased by 8°C/min to 200°C, and held at 200°C for 19 min, total time of 35 min).

The mass spectrometer was operated in the electron impact ionization mode at 70 eV; the mass range used was m/z 29 to 300. The mass spectrometer interface and ion source temperatures were 210°C and 200°C, respectively. The VOCs were identified by comparison with the mass spectra of the National Institute of Standards and Technology (NIST) 11 Mass Spectral Library (NIST, Gaithersburg, MD). The peak of the VOC was integrated from specific ions for each molecule to avoid overlapping between compounds.

Retention of fungal lipase onto the experimental curds

Experimental curds were prepared using skim milk, which contained neither milk lipid nor lactic starter culture. Five kilograms of skim milk were heated at 72°C for 15 sec and cooled down to 31°C. Lactic acid solution was then added to adjust the pH to 5.5. After the addition of 0.3 g rennet (Chr. Hansen, Hoersholm, Denmark) dissolved in 0.25 mol L⁻¹ NaCl, subsequent procedures were the same as the cheesemaking. The curds were stored at -20°C until use.

As illustrated in Supplementary Figure 1, two test tubes containing the FDP (40 mg) of each strain dispersed in 4 mL simulated milk ultrafiltrate (SMUF)¹⁵ pH 5.5, were prepared and pre-incubated in a water bath at 30°C for 10 min. Either of them received

4 g of the skim milk curds. Subsequently, both samples were homogenized (IKA® T25 disperser with S25N-8G tool, Germany) at 15,000 rpm for 3 min, followed by incubation at 30°C for 30 min. Then the tubes with curds were filtered through coarse filter paper (Azumi, Japan) and both samples were centrifuged at $21,130 \times g$ at 25°C for 15 min. Three hundred microliters of the recovered supernatant were added to 2 mL of the substrate and incubated at 30°C for 30 min to determine lipase activity. In this experiment, butter oil was replaced with olive oil as the substrate.

The whole enzyme from *A. oryzae* AHU 7139 was further fractionated into extracellular and intracellular enzymes. The extracellular enzyme was extracted by adding an equal weight of deionized water to CP and centrifuged at $21,130 \times g$, at 4°C for 10 min. The precipitates were dispersed in deionized water to extract the remaining extracellular enzyme. This additional extraction was performed twice, and these extracts were pooled to use as the extracellular enzyme. The precipitate was washed with deionized water once again and centrifuged at $21,130 \times g$ at 4°C for 10 min. Then, the precipitate was freeze-dried and ground. A suspension of the freeze-dried powder in SMUF was used as the intracellular enzyme. To fractionate the intracellular enzymes, the freeze-dried powder was dispersed in SMUF at 30°C for 30 min and centrifuged at $21,130 \times g$ at 4°C for 10 min to recover the supernatant, which was used as the water-soluble intracellular enzyme. The precipitate was washed by deionized water three times to remove any remaining water-soluble intracellular enzyme. Then, the precipitate was suspended in SMUF to use as the insoluble enzyme. Thus, three enzymes, namely from the extracellular, intracellular soluble, and insoluble fractions, were used in the presence or absence of skim milk curds in the same manner shown in Supplementary Figure 1.

Analysis of SMUF extractable peptides from skim milk curds by SDS-PAGE

To observe the curd components relevant to cell-binding lipase solubilization, 1 g of the skim milk curds was mixed with 1 mL of SMUF pH 5.5 to homogenize (IKA® T25 disperser with S25N-8G tool, Germany) at 15,000 rpm for 3 min followed by filtration. The filtrate was subjected to SDS-PAGE using 12.5% polyacrylamide gel.¹⁶ Whole casein (0.2% casein in 0.05 mol L⁻¹ Tris-HCl buffer pH 7.0) and commercial protein standard (DynaMarker® MultiColor III, Biodynamics laboratory, inc., Tokyo, Japan) were also loaded, and the gel was stained with Coomassie Brilliant Blue R-250.

Statistical analysis

The composition of water, salt, total protein, total lipid, WSN in total nitrogen, and FFA content in the cheeses were analyzed using Tukey-Kramer's multiple comparison test. The data were analyzed by JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $p < 0.05$. The difference in the amounts of VOCs in cheese samples made using different adjuncts from *Aspergillus* strains was analyzed using a principal component analysis (PCA).

RESULTS

Lipase and protease activity in adjunct materials

The amount of each culture FDP for 1 kg of cheesemaking was adjusted to an equivalent level of lipase activity (Table 1), so the initial protease activity was variable depending on the adjunct materials. The highest protease was supplied through the adjuncts from *A. oryzae* with 85.0 PU, which was 4.6 times more than that of *A. kawachii* (18.5 PU).

Cheese composition

After 12 weeks of ripening, all the experimental cheeses were analyzed. The results of the chemical analysis of the experimental cheeses are shown in Table 2. The moisture, salt, lipid, and protein content were not significantly different between the four types of cheese. However, every *Aspergillus* adjunct caused a higher FFA level, in particular, the *A. oryzae* adjunct resulted in a significantly higher amount of FFA compared to the other three types. In contrast, WSN amounts increased in a dose-dependent manner with proteolytic activity in these three adjuncts.

Volatile organic components

VOC levels were evaluated using an SPME technique and the values were expressed as the peak area. Then PCA was performed to show the relative VOC amounts in the cheese samples (Figure 1). Thirty-four volatile compounds were detected in the experimental cheeses and were categorized into 6 different compound groups, including 11 FFA, 7 ketones, 7 hydrocarbons, 4 ethyl esters, 3 alcohols, and 2 aldehydes. Although all 34 VOCs were found in each of the samples, the dominant VOC levels differed with each adjunct used. The control cheese had high amounts of acetoin, toluene, and alcohols such as ethanol, 1-octanol, and 2,3-butanediol, while the *A. kawachii* and *A. luchuensis* cheeses had higher proportions of ketones, including acetone, 2-pentanone, and 2-nonanone, and 3-methyl-butanal. Benzaldehyde, 2-undecanone, FFA, and ethyl ester amounts were higher in the *A. oryzae* cheese than in the others. Accordingly, these *Aspergillus* adjuncts provided their characteristic VOCs in the resulting cheese products.

Retention of fungal lipases onto the experimental curds

Table 3 shows the distribution of lipase activities in the presence or absence of the skim milk curds using the whole enzyme or its fractions dispersed in SMUF. Lipase activity of *A. kawachii* was reduced from the initial of 601.1 LU to 399.2 LU after incubation with the curds, which implies that about 30% of the lipase was entrapped in the curds. In contrast, the initial lipase activity of *A. luchuensis* (307.9 LU) was maintained after incubation with the curds (313.6 LU), which shows the negligible affinity of *A. luchuensis* lipase towards the skim milk curds. It was of interest to note that the unexpected activation was recognized in *A. oryzae*, as its lipase activity increased by 7.2 times.

Because of the remarkably high FFA in *A. oryzae* adjunct cheese and the pronounced lipase activation after incubation with curds, *A. oryzae* was selected for further study to identify the related fraction. Fractionation of *A. oryzae* FDP revealed that the intracellular fraction was more obvious in terms of activation ratio after incubation with the curds compared to the extracellular one (6.4 and 1.7 times, respectively). Since the intracellular fraction comprises soluble and insoluble components, they were fractionated into cytosolic soluble and insoluble fractions. The degree of the activation of the intracellular soluble fraction was comparable to that of the extracellular one. In contrast, the degree of activation of the intracellular insoluble fraction was unavailable since lipase activity was negligible in the recovered supernatant of the insoluble fraction. When the insoluble materials were suspended in SMUF with no incubation with the curds to evaluate its lipase activity, 57.6 LU of the activity was detected, which showed that the lipase activity being attached to the cell components was lower than intracellular soluble lipase activity (72.0 LU). However, incubation of the insoluble fraction with the curds caused the release of the lipase from the cell components into the

supernatant, and the recovered activity was 244.8 LU, which was as much as 4.3 times of the activity on the cells (57.6 LU).

Analysis of extracted skim milk curd peptides

The above results strongly suggested that the release of the cell-binding lipases of *A. oryzae* relied on the soluble components in the skim milk curds. Thus, SDS-PAGE of the curd extract using SMUF was performed to observe the proteinaceous profile. Despite high hydrophobicity of milk curds, numerous peptides were extracted and recovered in the soluble fraction by the addition of SMUF, as shown in Figure 2. Polypeptides migrated faster than the intact caseins were detected, which implied that they were derived from casein degradation. Two faint bands representing higher molecular mass than the intact caseins were estimated to be 66 and 70 kDa and assumed to be serum albumin and lactoperoxidase, respectively.

DISCUSSION

Since the lipases in dairy products can cause rancidity, it is crucial to select proper *Aspergillus* when they are attempted to be used as the cheese adjuncts. Despite addition of equivalent lipase activity prepared from three sorts of *Aspergillus* adjunct materials, a significantly higher FFA level was found in the *A. oryzae* cheese. Therefore, we attempted to clarify the reason for the different levels of FFA in the resulting products from the point of view of the lipase affinity with the curds using laboratory-scale prepared skim milk curds. Through focusing on the lipase fractions from *A. oryzae*, the possible mechanism behind the higher level of FFA in its adjunct cheese was postulated.

At the first step, the adjunct materials with an equivalent lipase activity were prepared using three species of *Aspergillus* strains to produce cheese and the FFA levels of the resulting products were compared. We determined the lipase activity in the three

FDP adjuncts under the acidic and low-temperature condition using butter oil as a substrate to simulate conditions close to cheese ripening. Since the higher lipolytic activity was detected in the culture products from *A. kawachii* and *A. luchuensis* than that of *A. oryzae*, the amounts of FDP adjunct for these two experimental cheeses were reduced. *A. kawachii* and *A. luchuensis* have been used as the starter koji-mold for making shochu. Accordingly, attention has been paid solely to amylase and glycosidase rather than to protease and lipase productivity.^{17,18} However, comparable or even higher protease and lipase activity for *A. kawachii* have been found in rice-koji for Doenjang making, compared to *A. oryzae*.¹⁹ Thus, the species other than *A. oryzae* were thought to be deserved to be applied for cheese adjunct preparation. As expected, the degree of WSN generated was entirely consistent with the protease activity involved in the adjunct materials showing the feasibility of these adjunct materials to promote proteolysis as was shown in our previous study.⁵ *A. luchuensis* adjunct materials provided a comparable level of WSN to *A. oryzae* whereas lower FFA levels as compared to the control cheese, which suggests the potential for the development of rancidity free with higher proteolysis cheese. Although it is a beyond scope of this study, further comparison of the peptides and free amino acids profiles would clarify the traits of the two adjunct cheese more specifically because undesirable proteolysis could cause generation of bitter taste. On the contrary, the higher impact of the *A. oryzae* adjunct material on FFA content was confirmed despite the supplement of equivalent activity of lipase. Furthermore, the volatile compound analysis clearly showed that the *A. oryzae* cheese produced FFAs in addition to ethyl esters, benzaldehyde, and 2-undecanone, whereas the major odor components found in the *A. kawachii* and *A. luchuensis* cheeses were acetone, 2-pentanone, 2-nonanone, and 3-methyl-butanal, which was similar to be found in the well-known mold cheese using *Penicillium roqueforti* and *P. camemberti*,

which provide a high concentration of methyl ketones, mainly 2-nonanone and 2-heptanone in blue and Camembert cheese.²⁰ Thus, characteristic traits of cheese were able to be developed using the adjunct material from these three strains in terms of proteolysis, lipolysis, and odor profile.

Our original assumption of the high FFA accumulation in the *A. oryzae* cheese product was a high level of fungal lipase retainability onto the curd matrix. In this context, the lipase from *A. oryzae* was hypothesized to be more adsorbable onto the curds, while that from *A. kawachii* and *A. luchuensis* was not expected as much. In this cheese manufacture protocol, the adjunct materials were mixed with curds followed by pressing, which drained the whey. Accordingly, we introduced *in vitro* simulation to evaluate how much lipase activity would be drained away with whey. If the ratio of the lipase entrapped in the curds is higher in *A. oryzae* than *A. kawachii* and *A. luchuensis*, lower lipase activity should be remained in the soluble fraction after incubating with the curds *in vitro*. However, high activation was contrarily recognized in *A. oryzae* lipase after incubation. Even though the skim milk curds were replaced with the conventional cheese curds containing milk lipids and lactic starter, this activation of *A. oryzae* lipase was confirmed as well, which suggests that milk lipids and lactic acid bacteria are not concerned in this activation (data not shown). Furthermore, the affinity of *A. kawachii* lipase toward cheese curds was noted, despite no remarkable FFA increase having taken place in the *A. kawachii* cheese. Based on these conflicting results, it might be concluded that the higher accumulation of FFA in the experimental cheese might not depend on an affinity of the lipase onto cheese curds, and another explanation must be proposed.

Then, we focused on the remarkable lipase activation of adjunct material from *A. oryzae* AHU 7139 after incubation with the curds. The lipases involved in the adjunct

materials from *A. oryzae* AHU 7139 were categorized into three types: extracellular, intracellular-soluble lipase, and intracellular-insoluble lipase that was regarded as the cell-binding lipase. As shown in Table 3, the extracellular lipase took the highest ratio, followed by the intracellular-soluble and the cell-binding fraction in the absence of contact with the curds. However, incubation with the curds caused the activation of every fraction, in particular, the cell-binding fraction. Furthermore, this study demonstrated that the lipase originally bounded on the cells, as the insoluble form was turned to be soluble through the incubation with the curds. In this regard, it might make us underestimated the lipolytic potential when we decided the adjunct amount for the cheese manufacture because we had not noticed such kind of release and activation of the cell-binding lipase due to the curds. Several extracellular lipases of *A. oryzae* have been studied,²¹ and we confirmed that *A. oryzae* AHU 7139 produces at least two types of extracellular lipases by the column chromatography, one of which was identified as the known diacylglycerol lipase with no specificity towards triglycerides.²² The production of a diacylglycerol lipase is not specific for *A. oryzae* because *A. kawachii* and *A. luchuensis* produce this type of lipase as well.^{17,23} However, digestibility of the adjunct material from *A. oryzae* AHU 7139 towards purified diolein was 3.3- and 3.5-fold as high as that from *A. kawachii* NBRC 4308 and *A. luchuensis* RIB 2604, respectively (data not shown), showing that the capability of degradation for the subsequent substrate of diglycerol in adjunct material from *A. oryzae* AHU 7139 was much higher than that from other two strains. Thus, it can be concluded that the higher digestibility of diacylglycerol lipase in *A. oryzae* together with triacylglycerol lipase possibly promoted the high lipolysis in the ripening cheese. In contrast, limited information has been available regarding the cell-binding lipases of this species. Considering its unique responsibility towards the curd components, further observation

of cell-binding lipases is needed to investigate their interaction with the c:and subsequent release from the cells, in addition to their molecular characterization and/or location in the fungal cells.

According to the general understanding, cell-binding protein can be solubilized with surfactant. In contrast, skim milk curds contain neither surfactant nor emulsifier, while it contained calf rennet comprising chymosin as the major protease and pepsin as the minor one. Thus, we first assumed that the addition of rennet solution to the insoluble fraction allows the release of the lipases into the soluble fraction. However, no lipase activity was found after treatment of cell binding fraction with rennet solution without curds (data not shown). Then, we focused on what proteinaceous components in the curds were extracted accompanied by the solubilized cell-binding lipase. Chymosin specifically cleaves the Phe105–Met106 bond in bovine milk κ -casein²⁴ in the early stage of the standard cheese-making protocol. However, at the later stage, the pH of the curd drops due to the growth of the lactic starter. Since chymosin exerts its activity under acidic conditions of pH 3 – 6,⁶ it could turn out to be active on the other casein components, in addition to κ -casein. In this study, the skim milk curds were prepared at pH 5.5, which is close pH circumstance at the beginning of the cheese ripening and we found several peptides in their extracts on SDS-PAGE. Most of them were considered to be from α_{s1} -casein and β -casein, which are major components in the skim milk curds. Green²⁵ reviewed cleavage sites of chymosin towards α_{s1} -casein and β -casein under acidic conditions. Both are amphiphilic,^{26,27} and some of their peptides have been shown to exhibit emulsifying properties.^{28,29} Thus, these casein-derived peptides could work as surfactants and release the lipases attached to the cells into the soluble fraction.

Overall, we postulated a flow of the elevated FFA in *A. oryzae* cheese, as illustrated in Figure 3. In the adjunct preparation step, The pressing procedure prompted draining

the extracellular and intracellular soluble lipases with whey, while the cell-binding lipases from the FDP adjuncts were released from the fungal cell-matrix and turned into a soluble form in the cheese block due to the curd-derived peptides, which are relevant to the activation of the lipase as well. Consequently, the released and activated cell-bounded lipases along with the extracellular lipase responsible for triglycerides that remained in the curds degrade the intact milk lipids as the first step. Then, the resulting diglycerides were supplied to the subsequent substrates for diacylglycerol lipase as the second lipolysis step. These consequences are likely to be the cause of the elevated FFA in the *A. oryzae* cheese and produce an excess amount of volatile fatty acid over the threshold, leading to rancid flavor in *A. oryzae* cheese products.

In conclusion, the progressed lipolysis in the *A. oryzae* adjunct cheese was attributed to synergic effect of the predicted extracellular lipase responsible for triglycerides and the cell-binding lipases followed by diacylglycerol lipase that was lacking digestability towards triglycerides. This study uncovered the significance of cell-binding lipase when the culture products of *Aspergillus* were applied as adjunct materials for cheese flavor enrichment. Furthermore, curd-derived peptides were proven to be concerned in FFA accumulation of the *A. oryzae* cheese. Our finding will pave the way to improve cheese adjunct materials using genus *Aspergillus* as the rancid-free cheese flavor enrichment.

ACKNOWLEDGEMENTS

The authors are very grateful to Yu Toba and Hiroki Matsumo, Field Science Center for Northern Biosphere, Hokkaido University, for their assistance in cheese manufacturing.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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512

Table 1. The amount of adjunct FDP used for 1 kg cheesemaking and the initial LU and PU				
Adjunct recipe	FDP weight (g)	UNFDP weight (g)	Total LU	Total PU
Control	0	2.16	0	0
<i>A. kawachii</i>	0.70	1.46	666.6	18.5
<i>A. luchuensis</i>	1.09	1.07	666.6	50.0
<i>A. oryzae</i>	1.38	0.78	666.6	85.0
FDP: freeze-dried powder, UIFDP: un-inoculated freeze-dried powder				

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Table 2. The chemical analysis of experimental cheeses after ripening						
Cheese sample	Moisture (%)	Salt (%)	Lipid (%)	Protein (%)	FFA (mmol kg⁻¹)	WSN (%)
Control	33.0 ± 0.2	1.3 ± 0.0	30.5 ± 0.1	29.5 ± 0.3	295.5 ± 0.1a	11.9 ± 0.2a
<i>A. kawachii</i>	32.5 ± 0.2	1.4 ± 0.0	31.6 ± 0.5	29.2 ± 0.0	332.9 ± 0.1b	12.5 ± 0.2a
<i>A. luchuensis</i>	32.6 ± 0.3	1.3 ± 0.0	31.8 ± 0.2	28.5 ± 0.2	331.9 ± 0.5b	16.7 ± 0.0b
<i>A. oryzae</i>	33.3 ± 0.2	1.3 ± 0.1	29.6 ± 0.2	29.0 ± 0.8	494.7 ± 0.1c	17.8 ± 0.4b
Values are mean ± SE (N=2). a, b and c within a column indicate a significant difference between the samples in each component at p < 0.05.						

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Table 3. Distribution of fungal lipase activity (LU) before and after incubation with skim milk curds.					
Strain	Fraction		Skim milk curds		
			Before ^A	After ^B	B/A
<i>A. kawachii</i>	whole		601.1	399.2	0.7
<i>A. luchuensis</i>	whole		307.9	313.6	1.0
<i>A. oryzae</i>	whole		100.5	720.0	7.2
	whole	extracellular	237.6	405.0	1.7
		intracellular	124.2	799.2	6.4
	intracellular	soluble	72.0	127.8	1.8
		insoluble	trace	244.8	-
B/A is the ratio of lipase activity before and after incubation with skim milk curds <i>in vitro</i> . 1 LU= 1 μmol oleic acid/h/g-CP					

525 **Figure Legends**

526 **Figure 1.** Principal component (PC) analysis biplot (PC 1 and 2) of VOC attributes of
527 experimental cheeses. Numbers represent: 1–2 = control cheese, 3–4 = *A. kawachii*
528 NBRC 4308 cheese, 5–6 = *A. luchuensis* RIB 2604 cheese, 7–8 = *A. oryzae* AHU 7139
529 cheese.

530 **Figure 2.** SDS-PAGE patterns of the proteins from intact acid casein and extracted
531 skim milk curds; M: molecular mass marker protein; lane 1: intact acid casein; lane 2:
532 extracted skim milk curd soluble fraction in SMUF buffer.

533 **Figure 3.** The proposed flow of free fatty acid (FFA) accumulation in *A. oryzae* cheese.
534 Freeze-drying and grinding fragmented the cell walls of *A. oryzae* mycelia. The powder
535 is mixed with cheese curds followed by a pressing step. Pressing of the cheese allowed
536 flowing out some extracellular and intracellular-soluble lipases with the whey, while the
537 cell-binding lipases can be released from cell debris by curd-derived peptides. All the
538 retained lipases on cheese curds are activated by curd-derived peptides; they allow FFA
539 accumulation due to lipolysis during ripening period.

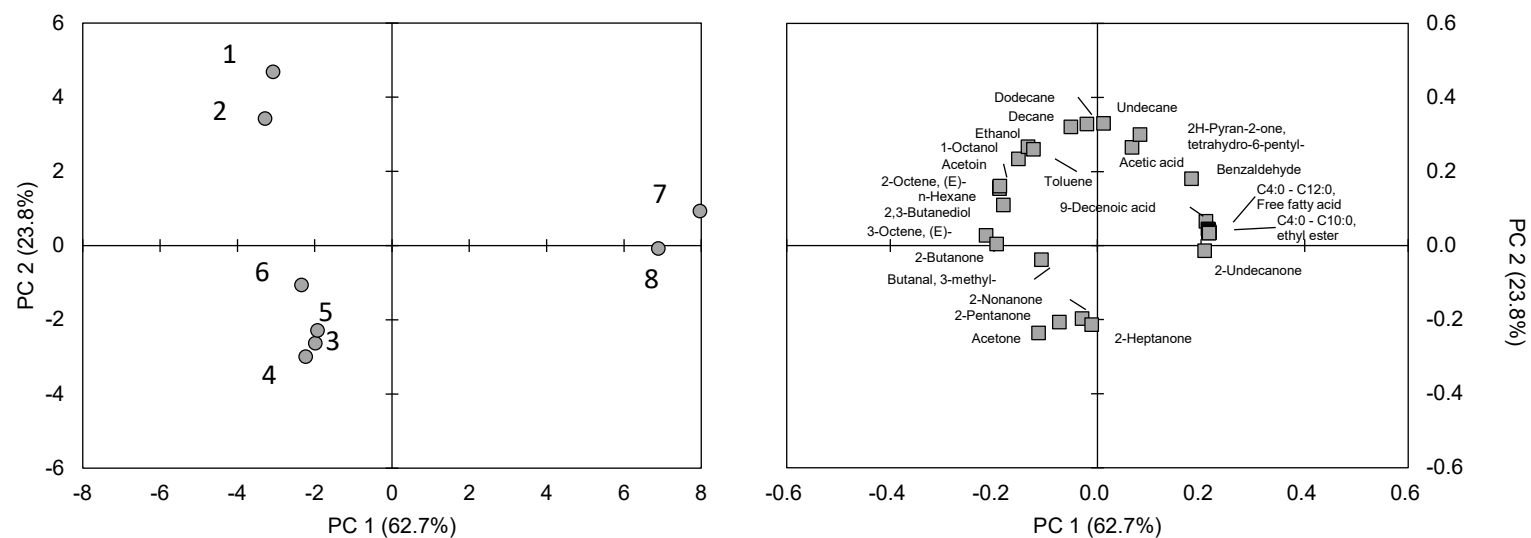


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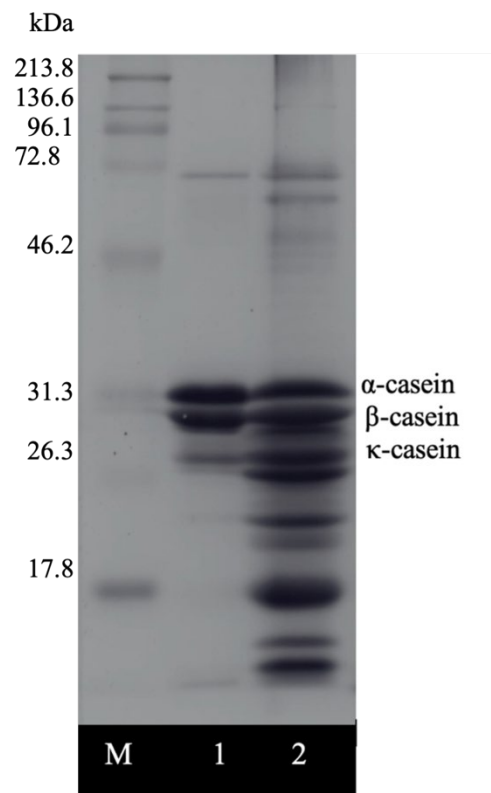


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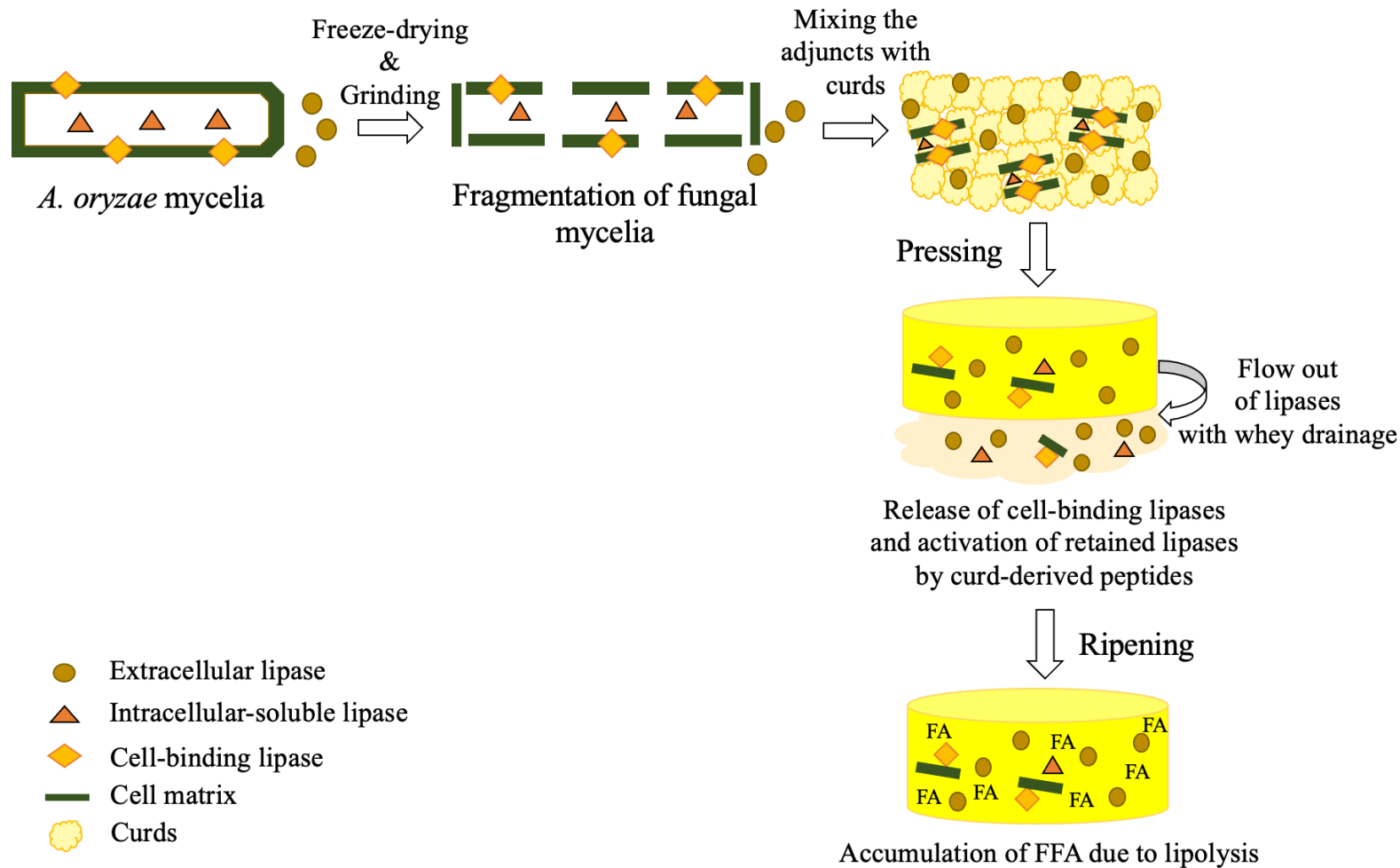
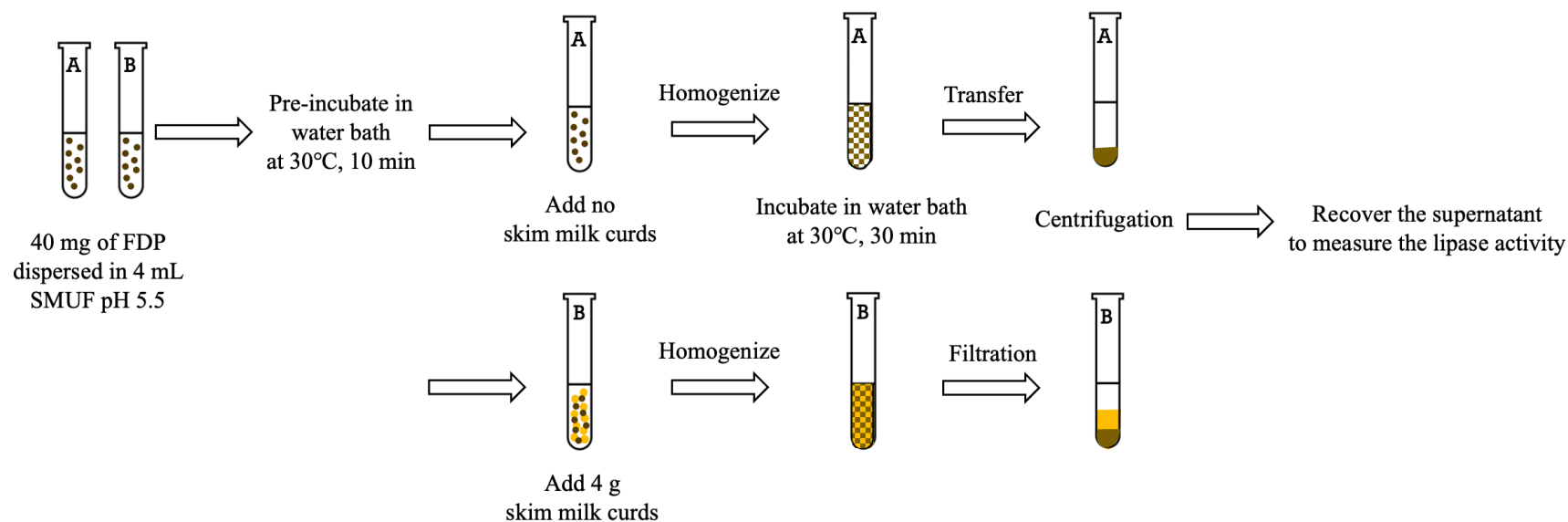


Figure 3. The proposed flow of free fatty acid (FFA) accumulation in *A. oryzae* cheese. Freeze-drying and grinding fragmented the cell walls of *A. oryzae* mycelia. The powder is mixed with cheese curds followed by a pressing step. Pressing of the cheese allowed flowing out some extracellular and intracellular-soluble lipases with the whey, while the cell-binding lipases can be released from cell debris by curd-derived peptides. All the retained lipases on cheese curds are activated by curd-derived peptides; they allow FFA accumulation due to lipolysis during ripening period.



Supplementary Figure 1. Flowchart of the evaluation of lipase distribution in the presence of experimental skim milk curds. When the fractionation was performed, FDP was replaced with fractionated enzymes and mixed with the SMUF