博 士 論 文 の 要 約

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学 位 論 文 題 名

Studies to reduce rancidity in ripened cheese caused by koji adjunct from *Aspergillus oryzae*

Introduction

Aspergillus oryzae is an edible filamentous fungus that has been used in traditional Japanese fermented products for a long time. However, *A. oryzae* is rarely used in cheese manufacture. The application of *A. oryzae* for cheese making is attractive due to its high proteolytic activity that could enrich cheese flavor. Nevertheless, once the extensive lipolytic reaction occurs leading to the release of volatile free fatty acids (FFA) from milk fat over the threshold, off-flavor called rancid could be caused.

Referring to an example of white and blue mold-type cheese using *Penicillium camemberti* and *P. roqueforti*, Nakanishi and Nakazawa (1965) first attempted to let *A. oryzae* grow on the cheese surface to accelerate ripening following the Gouda-type cheese production. Kataoka *et al*. (1987) found bitterness when attempting to accelerate the ripening of Gouda and Fedrick *et al*. (1986) also found it in cheddar cheeses using protease extracted from *A. oryzae*. Thus, the addition of commercial enzyme preparation from *A. oryzae* to promote ripening has often ended up with unsatisfied results. Nevertheless, several decades later, a cheese with koji fungi growing on the surface has launched in May 2021 under the name of “KURA koji cheese” (Zao dairy company). This is the world's first cheese aged by koji mold supplied with sake lees. The product contains more than five times glutamic acid, one of the umami ingredients, than Camembert cheese and no bitter flavor has been noticed (Biotechnology Research Support Center Website, 2021). In contrast at about the same time, Kumura *et al*. (2017) attempted another way to apply *A. oryzae* as a koji adjunct for cheese making, taking the high proteolytic potential of *A. oryzae* into account, as it was assumed to encode 135 proteinase genes through whole genome analysis (Machida *et al*., 2005). They used whey protein concentrate (WPC) as a solid substrate for *A. oryzae* and after suitable cultivation, the resulting products were directly mixed with fresh cheese curds prepared under the procedure for Gouda-type cheese making. In three months of ripening, the water-soluble nitrogen (WSN) amount, an index of cheese maturity, was increased in the cheese products by adding the culture products (CP) of *A. oryzae*. The increase of WSN amount was assured by Chintagavongse *et al*. (2022) when applying freeze-dry products (FDP) of *A. oryzae* growing on WPC in the same procedure. However, applying *A. oryzae* adjuncts caused a remarkable increase of FFA in some cheese products, in particular *A. oryzae* AHU 7139. Although proper lipolysis provides characteristic and favorable flavor from volatile compounds, excess accumulation of volatile fatty acids during cheese ripening causes undesirable flavor defect known as rancidity. Thus, it was turned out that *A. oryzae* would be applicable for cheese making if lipolytic action could be regulated properly. Thus, the rancid-related lipase and how to reduce rancidity in cheese products supplied with AHU 7139 koji should be studied.

This study aimed to elucidate the rancid mechanism specifically focusing on the rancid-related lipase genes in *A. oryzae* AHU 7139 and to establish the protocol of cheese adjunct preparation that allows limited rancidity in the cheese products. An *in vitro* curd experiment was conducted with adjuncts from some mutants (*ΔtglA* and *ΔmdlB*) selected

through the pre-screening of 25 annotated lipase genes using a molecular biological technique along with lipase activity measurement. Then, the effects of sugar as a sole carbon source on enzyme production were investigated between lactose in conventional WPC media and glucose in a modified whey-based medium (WPI+glucose) started at the acidic or neutral pH for cultivation. Moreover, the relationship between lipase genes and *farA*, which is considered to regulate lipase genes, was evaluated by quantitative PCR analysis. Lastly, the chemical analysis of the cheese products containing improved adjuncts of AHU 7139 with lower lipase activity was carried out. This study will provide valuable information to screen *Aspergillus* strains and establish the proper adjuncts preparation for a rancid-free cheese flavor enrichment.

1. Identification of rancid inducible lipase

Bioinformatics screening for rancid-related lipase

A. oryzae AHU 7139 was inoculated on whey solid medium whose pH was adjusted to 4.0 or 6.5 by lactic acid or NaOH solution and cultivated at 20°C for 7 days. The solid culture products (CP) were recovered and immediately freeze-dried and ground with a mortar and pestle to obtain freeze-dried powder (FDP). Total RNA was extracted and performed RT-PCR with design primers of 25 candidate lipase genes from *A. oryzae* database (FungiDB). From bioinformatics screening along with lipase activity analysis, 5 candidate genes were selected (Table 1).

Table 1. Primers used for gene expression analysis.

Gene	Primer	Sequence (5' - 3')	Predicted amplified size (bp)	
			genomic DNA	cDNA
Triacylglycerol lipase (*tglA*) (AO090003001507)	Forward	CTTTCACGGAGCCCTTCCAC	957	765
	Reverse	CGTAAATTAGTTCGCAGCCGC		
Mono-, di-acylglycerol lipase (*mdlB*) (AO090701000644)	Forward	TGAGTAGACCCTGCGAAGCAC	1024	921
	Reverse	CGAATTAGCGCAATGGCAATCCAG		
Cutinase 1 (*cutL*) (AO090005000029)	Forward	GCTTTGCCCCAGGAAGAAT	987	834
	Reverse	ACTGACCTCCATCAAATGGGAG		
AO090012000690 (1)	Forward	GAGATTTGATCGCACTGATGGC	1003	864
	Reverse	GCCAAGCTTTTGGCTTTCAC		
AO090005001319 (19)	Forward	CAGCCCACCCCCTTCAATAG	918	918
	Reverse	ACGGAACGATTCTTGACGAAAAC		

Disruption of individual genes in *A. oryzae* AHU 7139

Five candidate genes (No.1, No. 19, *mdlB*, *tglA*, and *cutL*) were selected to evaluate the lipase activity after gene disruption by split-marker system (Fig. 1).

Prior to the candidate gene disruption, the *pyrG* gene (AO090011000868) was knocked out in AHU 7139 by homologous recombination under selective pressure by 5-fluoroorotic acid (5-FOA). The *pyrG* knockout was aimed at conferring the uracil auxotrophy to AHU 7139 so as to make the *pyrG* available as a selectable marker. The DNA cassette for *pyrG* knockout was prepared by fusion. The resulting 2035 bp-long DNA fragment was purified by gel extraction and then applied to the transformation of AHU 7139. Protoplasts of AHU 7139 were prepared as reported (Tamano *et al*., 2007) and used for the transformation.

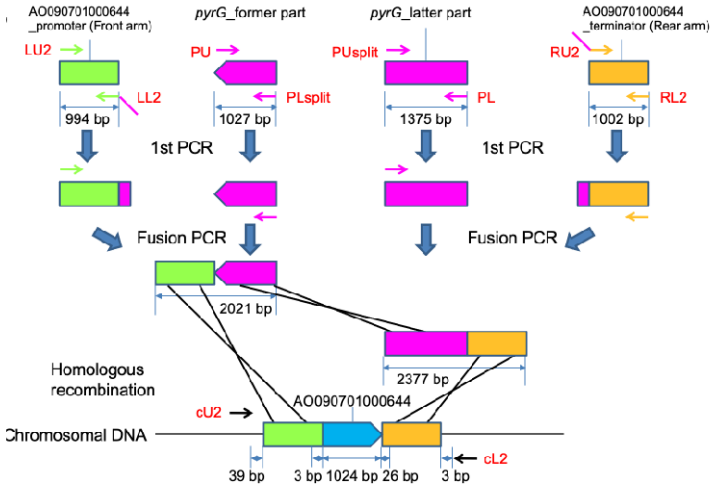

Fig. 1. Construction of the DNA fragment for disrupting AO090701000644 in *A. oryzae* AHU 7139 [pyrG$^-$]. The 2021 bp-long and 2377 bp-long DNA fragments were constructed for the disruption. Primers used for the construction and clone check are shown as colored and black arrows, respectively.

Then, five candidate genes were individually disrupted using AHU 7139 [pyrG$^-$] as a host. The candidate gene disruption was further confirmed by Southern hybridization. The acquired disruptants of the candidate genes (△1, △19, △*mdlB*, △*tglA*, and △*cutL*) and the parent strain were used to measure lipase activities and FFA levels in the *in vitro* cheese curd model.

Preparation of the extracellular and intracellular enzymes

The CPs were mixed with an equal weight of deionized water and treated by Exnizer® 400 homogenizer for food examination (Sansyo, Tokyo, Japan). The materials were then transferred to a centrifugal tube and centrifuged at 21,130 ×*g*, 4°C for 10 min. The supernatant was recovered, and the precipitate was re-extracted with deionized water another two times. The pooled supernatants were used as the extracellular enzyme. The precipitate was washed once more with deionized water and freeze-dried. The freeze-dried sample was ground into a fine powder with a mortar and pestle and then dispersed in a simulated milk ultrafiltrate (SMUF) at pH 5.5 (Jenness and Koops, 1962), which was used as the intracellular enzyme.

Measurement of lipase activity

The extracellular enzyme (70 µL) was mixed with 230 µL of SMUF buffer pH 5.5, while the suspension comprising 0.05 g of intracellular FDP dispersed in 300 µL of SMUF pH 5.5 was used as the intracellular enzyme. The lipase activity was determined as previously described (Kumura *et al*., 2019), using 1% purified triolein or diolein emulsified with 2% polyvinyl alcohol (degree of polymerization 2000) was used as the substrate. The substrate was divided into 2 mL for each test tube and subjected to pre-incubation at 30ºC for 5 min, followed by the addition of the enzyme to be tested (300 µL). The reaction was carried out at 30ºC for 30 min. The reaction was terminated by adding 7.5 mL of extract solution (heptane : isopropanol : 0.5 mol L^{-1} sulfuric acid = 48:48:4). Then, all tubes were vigorously shaken for 2 min and stood at least 1 h. The upper-phase solution was taken and measured free fatty acids amount according to the phenol-red method (Saito, 1979) using a spectrophotometer at 570

nm. Lipase activity was expressed as µmol of released oleic acid from the substrate per 1 h at the defined temperature from one gram of the CPs (1 µmol oleic acid/h/g-CP = 1 lipase unit: LU).

Introducing purified substrates revealed that the parent strain of *A. oryzae* AHU 7139 produced diacylglycerol (DG) lipolytic activity approximately twenty times higher than triacylglycerol (TG) lipase activity at both initial pHs (Fig. 2). The activity was predominantly found in the extracellular fraction although the CPs prepared with an initial pH of 4.0 showed a higher ratio of intracellular DG lipase than those prepared at pH 6.5. Reduced TG lipase activity was mostly observed in Δ*tglA* followed by Δ*mdlB* when cultivation was initiated at pH 6.5 compared to the parent strain. Thus, the predominant TG lipase activity was due to TglA in the culture initiated at pH 6.5. Regarding the DG lipase, Δ*mdlB* showed the greatest lipase reduction (84%) compared to the parent strain under either pH condition. Thus, major DG lipase activity could be ascribed to MdlB. Considering the higher level of total lipase activity in the culture initiated at pH 6.5, TglA and MdlB were more likely to be related to rancidity. Therefore, Δ*tglA* and Δ*mdlB* were selected for further study.

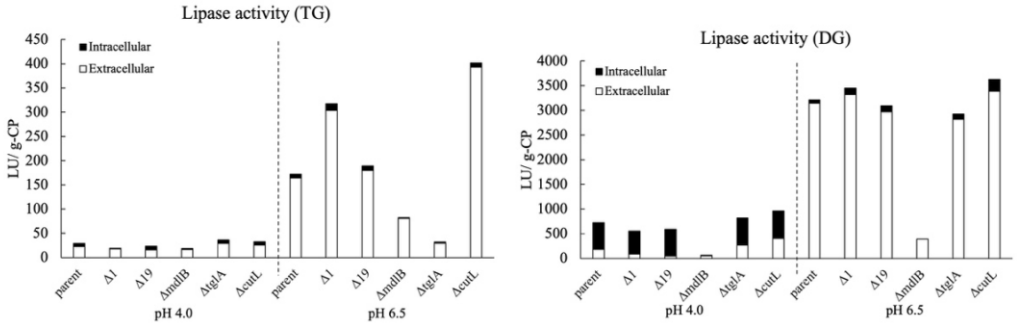

Fig. 2. Lipase activities of *A. oryzae* AHU 7139 (parent) and five selected mutants (Δ*mdlB*, Δ*tglA*, Δ*cutL*, Δ1, Δ19) toward purified triolein (TG) and diolein (DG) from the solid culture products (CP) initiated with pH 4.0 and pH 6.5. Values are expressed in average value (n = 2).

Study of the FFA levels in the *in vitro* curds using mutants

The *in vitro* cheese curds used in this study were prepared as previously described (Chintagavongse *et al*, 2022) with the addition of 2% NaCl. After producing the curds, 0.2 g of FDP prepared using the culture with an initial pH of 4.0 or 6.5 was mixed with 200 g of curds, and 20 g was divided into nine bags, and then vacuum-sealed. The culture with the parent strain (P4, P6.5) or mutants, including Δ*mdlB* (M4, M6.5) and Δ*tglA* (T4, T6.5), were used. The control cheese (C) received no adjunct material. The curd packs were ripened at 12ºC and sampled at 4 weeks.

For FFA determination, the triplicate curd sample (0.2 g) was transferred to 2 mL of 7.7 mol L^{-1} HCl in a test tube and placed into boiling water to dissolve the sample. FFA was extracted and determined as the same manner described in the lipase activity measurement and its content was expressed as millimoles per 100 g of curds using oleic acid as the standard.

Since the FFA accumulation in Δ*tglA* was significantly reduced in the ripening curds regardless of the initial pH (Fig. 3), it is suggested that TglA triggers hydrolysis leading to rancidity. Nevertheless, lipases No. 1, No. 19, and CutL should still be active in either CPs

from Δ*tglA* and Δ*mdlB*. However, the CPs from Δ*tglA* showed comparable FFA levels to the control, which demonstrated that those lipases were not as important as TglA under the cheese ripening circumstance. The reduction of the FFA levels of Δ*mdlB* indicates that MdlB lipase should not be overlooked when considering rancidity. Okumura *et al*. (1980) reported that mono- and diacylglycerol lipases, from *P. cyclopium* M1, more easily hydrolyzed acylglycerols than triacylglycerol lipase, and the synergistic action of mono- and diacylglycerol lipase with triacylglycerol lipase could accelerate lipolysis. Wong *et al*. (2021) found elevated FFA productivity in *A. oryzae* RIB40 when they applied the overexpressed strain AO090701000644 (*mdlB*) showing that MdlB might be involved in lipolysis and release of FFA. Based on these results, a possible flow of in ripened cheese caused by *A. oryzae* AHU 7139 culture products can be drawn as shown in Fig. 4. Extracellular TG lipase (TglA) is crucial for the degradation of TGs in milk in the first step of rancidity. Subsequently, the resulting DGs are provided as the substrate for MdlB. The dual effect of TglA, followed by MdlB, is likely to cause an excess amount of volatile fatty acids and rancidity in cheese products made with *A. oryzae* AHU 7139. Thus, when suitable strains of *A. oryzae* are screened with respect to lipase activity for the preparation of adjuncts for cheesemaking, a strict evaluation of TG activity should be performed.

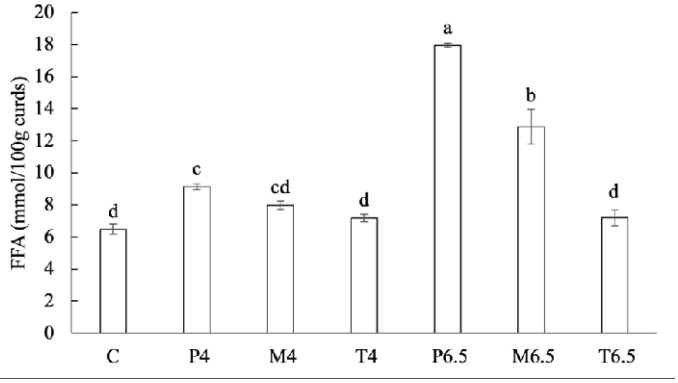

Fig. 3. Free fatty acid (FFA) profiles in cheese model at 4 weeks. C = curds only (control), P4 = parent AHU 7139 with initial culture pH 4, M4 = mutant AHU 7139 Δ*mdlB* with initial pH 4, T4 = mutant AHU 7139 Δ*tglA* with initial pH 4, P6.5 = parent AHU 7139 with initial pH 6.5, M6.5 = mutant AHU 7139 Δ*mdlB* with initial pH 6.5, T6.5 = mutant AHU 7139 Δ*tglA* with initial culture pH 6.5. Results of FFA level are expressed as the mean ± SE (n = 3). The different letters indicate a significant difference between the samples using Tukey-Kramer test ($p < 0.05$)

2. Effect of glucose on the expression of *farA* and lipase genes

Lipase activities and expression levels were reduced in the presence of glucose on the whey protein isolate solid medium (WPI+glucose) than on glucose-free WPC. FarA could be regulated by glucose and was hypothesized to positively regulate both TglA and MdlB while the regulating mechanism should be further investigated. CP of *A. oryzae* AHU 7139 on WPI+glucose with initial pH 4.0 was suggested for adjuncts to enrich flavor in cheese products due to high protease activity with limited lipase activity. Although the TG lipase activity was successfully reduced in this study, practical assessment was carried out using established CP from *A. oryzae* AHU 7139 in the following chapter to confirm its applicability.

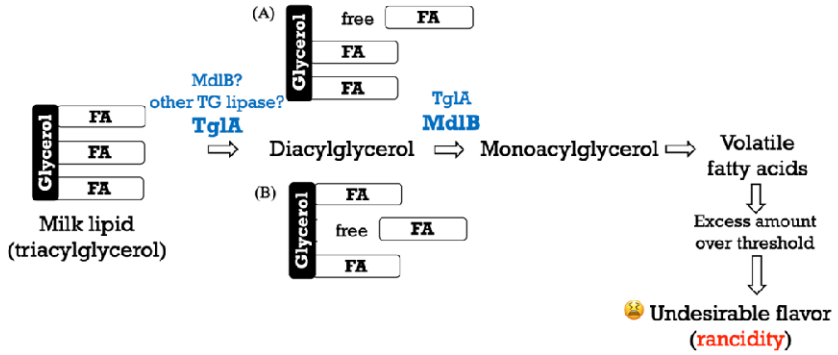

Fig. 4. The proposed rancidity process in the ripened cheese caused by *Aspergillus* lipolysis. As triacylglycerol (TG) is the major lipid in milk, triacylglycerol lipase (TglA) hydrolyzes TG at any fatty acid position ((A) sn-1, 3 or (B) sn-2) and generates diacylglycerol (DG) and free fatty acids (FAs) during ripening. Subsequently, diacylglycerol lipase (MdlB and, to some extent, TglA) hydrolyzes DG and produces FFA. The predominant lipases (TglA or MdlB) at each hydrolytic step are shown in bold, although other genes might be involved. The excess amounts of free volatile fatty acids released by fungal lipolysis could cause rancidity.

3. Evaluation of a cheese adjunct prepared on glucose-containing WPI culture for cheese flavor enrichment

Applying *A. oryzae* AHU 7139 adjuncts from whey-based CP improves the WSN levels in the ripened cheese. Furthermore, glucose addition in WPI medium enabled to reduce FFA level comparable to the non-adjunct control cheese. Therefore, the glucose addition into the medium for preparing *A. oryzae* CP as the adjuncts was proven worthwhile to avoid rancidity augmentation in cheese products.

Conclusion

RT-PCR analysis following *in vitro* curd experiments using established gene disruption mutants revealed that TglA triggers the degradation of milk fat followed by subsequent degradation of resulting diglycerides by MdlB. Moreover, the *in vitro* cheese curd experiment was proven to be more advantageous in predicting applicability of a koji as a cheese adjunct than TG lipase activity measurement, which was impossible to discriminate TglA activity from the TG lipase activity measurement because strain AHU 7139 produces several lipase molecules. Yet, the addition of glucose to WPI medium was proven to reduce *tglA* expression, which was likely to be due to the reduced expression of *farA* coding positive transcription factor of *tglA* and *mdlB*. CP of AHU 7139 on WPI+glucose with initial pH 4.0 was suggested for adjuncts to enrich flavor in cheese products due to high protease activity with limited lipase activity. Using this koji as an adjunct for cheese making resulted in a significant reduction of FFA levels in 3-month-cheese products compared to that using WPC. Moreover, WSN levels of all adjunct cheeses were significantly increased compared to the control.

Therefore, ceasing TglA production in *Aspergillus* koji might be a key point to limit the released volatile fatty acids from milk lipids to reduce rancidity. Either selection of TglA disfunction strains or adding glucose to the whey solid medium for *Aspergillus* koji adjunct preparation is beneficial to limiting rancid flavor in cheese products.

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