Attempt at the adjunctive use of solid-state culture products and its freeze-dried powder from *Aspergillus sojae* for semihard cheese

Short running title: Adjuncts as the raw and freeze-dried culture products of *A. sojae* for cheese

Napaporn Chintagavongse[1], Tomoki Yoneda[1], Chi Ming-Hsuan[2], Toru Hayakawa[1], Jun-ichi Wakamatsu[1], Koichi Tamano[3], Haruto Kumura[1,*]

[1]*Laboratory of Applied Food Science, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan;* [2]*National Taiwan Ocean University;* [3]*Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan*

* Corresponding author: Haruto Kumura

Mailing address: Laboratory of Applied Food Science, Graduate School and Research Faculty of Agriculture, Hokkaido University, 060-8589, N9, W9, Sapporo, Japan

Tel: +81-11-706-3642, E-mail: kumura@anim.agr.hokudai.ac.jp

Abstract

BACKGROUND: Some species belonging to the genus *Aspergillus* have been used in traditional Japanese fermentation foods. *A. sojae* is the species responsible for high proteolytic activity. Freeze-drying treatments followed by physical disruption enables the pulverisation of mycelia of *A. sojae* RIB 1045 grown in whey protein base solid media. Through this protocol, intracellular proteases were extracted to compare extracellular protease activity in terms of the reaction pH dependence in the presence or absence of the inhibitors.

RESULT: With different sensitivities to inhibitors, intracellular and extracellular proteases showed the highest activity under the acidic region, which was considered suitable for cheese application. The raw culture product (CP) and its freeze-dried product (FDP) were mixed with cheese curds prepared according to Gouda-type cheese making and were allowed to ripen for three months. Chemical analysis of the products showed 13.3% water-soluble nitrogen (WSN) in the control, which had received noncultured media, whereas 20.0% and 21.1% WSN were found in CP and FDP experimental cheese, respectively. Although these adjuncts significantly increased WSN, an insignificant difference was found between CP and FDP. Free fatty acids in all experimental cheeses were similar, showing that CP and FDP caused no rancid defects.

CONCLUSION: An introduction of freeze-drying treatments accompanied by cell disruption resulted in a negligible effect in terms of WSN. However, the application of *A. sojae* can be beneficial when it comes to increasing the degree of WSN compared with *A. oryzae*, as shown in our previous study.

Keywords: *Aspergillus sojae*; protease; solid media; cheese; water-soluble nitrogen

INTRODUCTION

Aspergillus oryzae and *A. sojae* are pivotal filamentous fungi that have been used in traditional Japanese fermentation products. *A. oryzae* secretes amylases and proteases that contribute to making sake (rice wine), shoyu (soy sauce) and miso (soybean paste). In contrast, *A. sojae* has been used solely for shoyu and miso fermentation because this species possesses high proteolytic activity while having a lower capability when it comes to starch saccharification. In part, this can be attributed to a single copy of the amylase gene, whereas *A. oryzae* possesses three copies of the corresponding genes, though both species are assumed to possess more than 130 proteolytic genes[1].

It is well-known that a solid culture is superior to liquid for enzyme production by filamentous fungi. In our previous study[2], whey protein concentrate (WPC) was used as a solid substrate for *A. oryzae*, and the CPs were mixed with fresh cheese curds prepared under the procedure for traditional semihard type cheese making. In three months of ripening, the water-soluble nitrogen (WSN) in the products increased with the addition of the CPs of *A. oryzae*. This protocol is advantageous because the autoclaving of a WPC solution readily leads to solidification because of the heat denaturation of whey protein. If necessary, the ingredients including other components and/or acidic or alkaline solutions for pH adjustment can be mixed with a WPC solution prior to autoclaving. Furthermore, additional treatments of the CPs that include freeze-dry followed by mechanical particleisation could disrupt the cell walls of filamentous fungi in the substrate. Adjunctive use of the CPs that had received this disruption procedure would allow not only for the action of the extracellular enzymes, but also the intracellular ones during ripening. It may be interesting to apply fungal intracellular enzymes for food development because the traditional Japanese fermentation protocol does not use this method.

In the current study, we determined the extracellular and intracellular protease activity of *A. sojae* with a brief characterisation. Subsequently, the experimental cheeses were produced using the CPs and its ground FDP to investigate the impact of the additional treatments during ripening.

MATERIALS AND METHODS

Strain and culture condition

The strain used in the current study was *Aspergillus sojae* RIB 1045, which was kindly gifted by National Research Institute of Brewing (Hiroshima, Japan). This strain was grown on potato dextrose agar (PDA; Merck KGaA) at 30°C for 10 days. Spore suspensions were prepared by adding a 9.0 g/L sodium chloride (NaCl) solution into the grown culture on PDA and then diluted by (9.0 g L^{-1}) NaCl solution to a concentration of 2.5×10^5 spores/mL, which was counted by using haemocytometer (NanoEntek, Korea).

Twenty-five grams of WPC80 (Fonterra, New Zealand) and 5 g of glucose was dissolved in 70 g of deionised water and adjusted to pH 4.0 with lactic acid. The solution was divided (10 g) to a 100 mL Erlenmeyer flask 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 the extracellular and intracellular enzymes

Figure 1 illustrates the enzyme preparation procedure. The CPs were mixed with an equal weight of deionised water and treated by a stomacher for 5 min. Then, the materials were transferred to a centrifugal tube and centrifuged at 21,130 xg, 4ºC for 10 min. The supernatant was recovered and used as the extracellular enzyme (fraction I). The precipitate was washed three times by deionised water and freeze-dried. The freeze-dried

sample was mechanically disrupted into a fine powder by mortar and pestle for 10 min and then dispersed in a 0.02 M sodium phosphate buffer pH 7.0 (20 mL g^{-1} FDP). This suspension was used as the intracellular enzyme (fraction II). Furthermore, fraction II was centrifuged at 21,130 xg, 4ºC for 10 min to obtain the supernatant as the water-soluble intracellular enzyme (fraction IIa). The precipitate in this step was washed by the 0.02 M sodium phosphate buffer pH 7.0 three times, and then, the sample was dispersed in 20 mL of 0.02 M sodium phosphate buffer pH 7.0 containing 1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) and let stand for 30 min. After centrifugation, the supernatant was recovered as the membrane-bounded fraction (fraction IIb).

Fluorecent microscope analysis

The CP and FDP specimens were prepared by placed a solid sample on the glass slide, added one drop of Calcofluor White Stain (Sigma-Aldrich, Canada), and then, one drop of 10% KOH (Kanto chemical, Japan) according to the manufacturer's instructions. Spreading sample and then close with a coverslip and left for 1 min. The physical appearance of the mycelia of CP and FDP were examined by the fluorescence microscope (Olympus BX-50). The Filter (DP50, U-PPMTVC) was used for DAPI excitation (Ex: 360-370, Em: 420-460). Photographs were taken by Viewfinder Lite snap camera program (2776 x 2074 pixels) connected to a computer.

Measurement of protease activity

The proteolytic activity was determined as previously described with some modifications[3]. The substrate, 0.2% casein, obtained by acid precipitation (pH 4.6) from raw skim milk, dissolved in 0.05 M sodium acetate buffer pH 5.5 (700 μL), was incubated with a desired enzyme fraction (50 μL) at 30ºC for 2 h. The reaction was terminated by adding 750 μL of trichloroacetic acid (TCA) (Wako, Japan) mixture; containing 0.11 M

TCA, 0.22 M sodium acetate and 0.33 M acetic acid, and further incubation was carried out for 15 min. The resulting mixture was centrifuged at 21,130 xg, 25ºC for 10 min. The supernatant (1 mL) was recovered and mixed with 2 mL of 0.625 M Na_2CO_3 followed by the addition of 0.7 N Folin reagent (Nacalai Tesque, Kyoto, Japan). The mixture was held at 30ºC for 30 min, and its absorbance at 660 nm was determined[2]. The value was corrected by subtracting that obtained from the blank, which was subjected to the same treatments except that a TCA reagent was added to the enzyme prior to the addition of the substrate. Enzyme activity was expressed as micrograms of the released tyrosine per h at 30ºC extracted from one gram of the CPs.

Characterisation of the protease activity

The extracellular enzyme fraction I was treated with 25–55% acetone precipitation, and the precipitates were dialysed against 0.02 M sodium phosphate buffer pH 7.0. The intracellular enzyme fraction of fraction IIa and fraction IIb were treated with 0–75% acetone and 25–66% acetone precipitation, respectively. The precipitates were recovered and treated as the same manner as fraction I.

To observe the effect of pH and inhibitors on the activity, 0.2% casein dissolved in 0.05 M buffer including sodium acetate pH 5.5, sodium phosphate pH 7.0 or Tris-HCl pH 8.5 were used in the presence or absence of the following five inhibitors (10 µg/µL Pepstatin A (Peptide institute, Osaka, Japan), 10 mM o-phenanthroline (o-phen) (Fujifilm, Osaka, Japan), 10 mM ethylene diamine tetraacetic acid (EDTA) (Kanto chemical, Tokyo, Japan), 10 mM phenylmethylsulphonyl fluoride (PMSF) (Nakalai Tesque, Kyoto, Japan) and 10 µM E64 (Peptide institute, Osaka, Japan), showing the final concentration during the reaction.

Preparation of adjunct materials for cheese making

The culture products were pooled and treated with a food processor to obtain fine particles (i.e., CP). As the control, an uninoculated solid medium was prepared in the same manner. For the freeze-dried sample, the CP was freeze-dried followed by mechanical disruption by mortar and pestle to obtain freeze-dried powder (FDP). From 1 g of the CP, 0.26 g of FDP was obtained.

Cheese making

A batch of cheese making was carried out in June 2019 per the conventional procedure of Gouda-type cheese making. Raw milk was obtained from the experimental farm in the Field Science Center for Northern Biosphere, Hokkaido University. The raw whole milk was standardised with the raw skim milk to adjust its fat content to 3.0% and heated at 72ºC for 15 sec. After cooling to 31ºC, the milk (97 kg) was transferred to a vat, and then, 1/1000 volume of 1.5 M $CaCl_2$ solution and 2% volume of bulk lactic starter (BD culture CH N-01; Chr. Hansen, Denmark) prepared in sterilised skim milk were added. After incubation for 60 min, 4.4 g of calf rennet (Chr. Hansen, Denmark) dissolved in 0.1 M NaCl solution was added to induce coagulation of the milk. After curd formation, it was cut into cubes of 10 mm^3 in size and left for 15 min. Subsequently, gentle stirring was performed at 31°C for 30 min, and 30 kg of whey was discarded. Then, 24 kg of warm water (60°C) was added to reach 39°C, followed by 15 min of agitation at this temperature. After the drainage, the curds were recovered and weighed to be mixed with 1% weight of the CP, 0.26% weight of the FDP or 1% weight of the uninoculated WPC solid culture (control) by hand uniformly to transferred to 1 kg size of mould for Gouda type cheese. These three types of cheeses were prepared in triplicate. Following brief pressure (1.0 kg/cm^2 for 10 min), inversion and the second stage of pressure (1.5 kg/cm^2

for 50 min) were performed. The curds were cooled in water overnight and treated with dry salting (20 g of NaCl per 1 kg cheese). The curds were matured at 11.5 ± 1ºC for 12 weeks with a relative humidity of 85 - 90%. After 15 days of the manufacture, the cheese surface was coated with wax (Paramelt, Netherland) and left for ripening.

Chemical analysis of cheese

All assays were performed in triplicate.

The moisture was determined according to the International Dairy Federation (IDF)'s recommendation[4]. Fat and protein were measured using the Association of Official Agricultural Chemists (AOAC) method[5,6].

WSN was determined according to the method of Kuchroo and Fox[7], with some modifications: a sample (5 g) was added to 25 mL of deionised water and, after treatment with a stomacher for 5 min, maintained at 40°C for 1 hr. The sample was centrifuged, and the supernatant was passed through glass wool to entrap lipids. The resulting filtrate was subjected to the micro Kjeldahl method[5].

After the extraction of the fat according to the AOAC protocol[6], free fatty acids (FFAs) were extracted by the phenol-red method[8]. Oleic acid was used as the standard and converted to the content of oleic acid (mmol) in 1 kg of cheese.

Statistical analysis

Comparisons between the proteolytic activity of the enzyme fraction against the inhibitors and pH effect were independently made using a one-way analysis of variance. Percentage of water, total protein, total lipid, WSN in total nitrogen and FFA content in the cheeses were analysed using Tukey–Kramer's multiple comparison test. The data were analysed by JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Effect of freeze-dry treatment on the CPs

Fluorescent microscope analysis was carried out to confirm disruption effect of *A. sojae* RIB 1045 in the freeze-dried culture products. Figure 2 showed that filamentous mycelia found in the raw culture products was broken into small pieces after the treatment of freeze-dry followed by grinding procedure. Thus, it was concluded that these treatments enabled to disrupt cells grown in the whey solid substrates.

Protease Activity

Because our preliminary study confirmed that the protease activity of fraction II (sum of IIa and IIb activities) was equivalent to 63% of the fraction I (data not shown), the cell disruption treatment was expected to boost the adjunct effect of the CPs for the cheese ripening because of the additional intracellular protease involvement. Subsequently, the extracellular and intracellular proteolytic feature was compared using fractions I, IIa and IIb. As shown in Table 1, all fractions showed the highest activity under the acidic condition, and the proteolytic activity of the neutral and alkaline conditions were comparable.

Despite the highest proteolytic activity under the acidic condition, PMSF exerted no inhibitory effect for fractions IIa and IIb at pH 5.5, whereas fraction I was sensitive to PMSF for all the pH conditions. Inhibition because of pepstatin A and EDTA was obvious in fraction I exclusively under the acidic condition. In contrast, pepstatin A and EDTA gave a significant inhibition to fraction IIb but not to fraction IIa at all pH conditions tested. In addition, all fractions were insensitive to o-phenanthroline and E-64.

Cheese Composition

The results of the chemical analysis of the experimental cheese is shown in Table 2. The water content was apparently similar between these three types of cheeses although a significant difference between CP and FDP was noted. In terms of protein, lipid and FFA, there were no differences between the three experimental cheeses. In contrast, it was evident that the WSN was increased by the addition of the adjunct materials. However, no significant difference of WSN was detected between the CP and FDP cheese.

DISCUSSION

Through our preliminary studies, we screened *A. sojae* RIB 1045 as a high proteolytic strain. Then, a freeze-dry treatment was used on the whey solid CPs of *A. sojae* RIB 1045, which successfully allowed for the extraction of the intracellular proteases. Furthermore, intracellular protease could be fractionated into water-soluble and Triton X-100 solubilised fractions, which here are fractions IIa and IIb, respectively. The latter was obtained only using the surfactant, suggesting that it was loosely bounded to cell walls or membranes. Focusing on the acidic pH circumstance of cheese, the respective protease activity in fraction IIb takes about 45% of the total intracellular fraction from the simple calculation of the results obtained in Table 1.

All fractions contained several protease species with different rational profiles, as shown in Table 1. Serine protease, which is sensitive toward PMSF[9], is likely to be involved, as confirmed in *A. oryzae*[10], *A. sojae*[11] and many kinds of *Aspergillus* strains[12]. As the acidic serine protease from *Aspergillus* sp., *A. oryzae* and *A. sojae*, aorsin is well-known[13]. Although pepstatin A inhibited protease activity[14] in fraction IIb in every pH condition, the inhibition was exclusive under the acidic condition in fraction I. Although aspartic protease of aspergillopepsin A (pepA)[15] or aspergillopepsin O (PEPO)[16] has been

known as belonging to this type of protease in *A. oryzae*, no information has been available in *A. sojae*. Furthermore, no report has been found regarding intracellular neutral and alkaline aspartic protease from *A. sojae*.

Fraction IIa contained neither serine protease nor metal protease, while in fractions I and IIb, acidic metalloprotease such as deuterolysin from *Aspergillus* sp.[17]was found. However, no intracellular neutral and alkaline metalloprotease has been reported in *A. sojae*. Thus, our study indicates that unidentified metalloprotease(s) and alkaline aspartic protease were included in fraction IIb. The inhibitory spectrum of fraction IIb was obviously different from fraction IIa, which implies another kind of protease that was being influenced by pepstatin A or EDTA under the alkaline condition and that could be supplied only if fraction IIb was involved for the working adjunctive materials. Nevertheless, there were no statistical difference in WSN between CP and FDP. We assumed that some loss in water-soluble intracellular protease because of the whey draining procedure during pressing. Accordingly, we might need to evaluate how much protease is retained in the curds after the pressing step. In this regard, the protease distributed in fraction IIb should have been more likely to be retained in the curds than in fractions I and IIa. However, if the disrupted materials kept a chopped-like structure, membrane or cell-wall attached proteases might have been less accessible to a solid protein substrate in the cheese. To make the membrane or cell-wall attached proteases more active, supplementary treatments might be needed such as use of edible chitinase and emulsifier to loosen the solid cell walls of fungi and improve the contact of the enzyme with the corresponding substances. Furthermore, effect of this disruption treatments should be investigated as well using other kind of cheeses manufactured such as different ripening period and water activity.

Although no statistical difference of WSN was found in the cheese using CP and FDP, the addition of those preparations led to 20.0 – 21.1% of WSN, which was higher than that of the control (13.3%) which represented the typical gouda-type cheese[2,18]. In our previous study[2], maximum WSN increase was recorded using the CPs of *A. oryzae* AHU 7139, whose WSN and its control was 14.7% and 12.5%, respectively. Furthermore, some of the resulting cheese products using CPs from *A. oryzae* AHU 7139 and *A. oryzae* AHU 7146 showed a remarkable increase of FFA compared with the control cheese, whereas no increase of FFA was found when *A. sojae* was used in the current study. In fact, we recognised neither bitter tastes nor a rancid flavour because of bitter peptide or volatile FFAs; however, we focused on chemical analysis rather than organoleptic assessment. These results suggest that application of *A. sojae* RIB 1045 into cheese is unlikely to cause the defects related to rancidness, and *A. sojae* would be more advantageous than *A. oryzae* here. In addition, our previous protocol introduced a filter on the solid whey substrate to obtain spore-free CPs through the removal of hyphae on the filter. However, we recognised no fungal growth during ripening in the current study despite using raw CPs with aerial hyphae. Thus, we can conclude that this separation procedure of aerial hyphae using the filter on the solid culture can be neglected.

In conclusion, an introduction of freeze-dry treatments accompanied by cell disruption gave less of an impact than we expected. However, further information is awaited regarding the efficiency of the freeze-dried materials as the adjunctive use for other type of cheese manufactured under different ripening period and water activity. Moreover, supplementary treatments with edible chitinase and emulsifier might be deserved to be involved. In further studies, it would be interesting to see which capabilities and potential could be exerted when the replacement of the raw CPs with

freeze-dried materials because their adjunct use for cheese ripening still needs to be examined.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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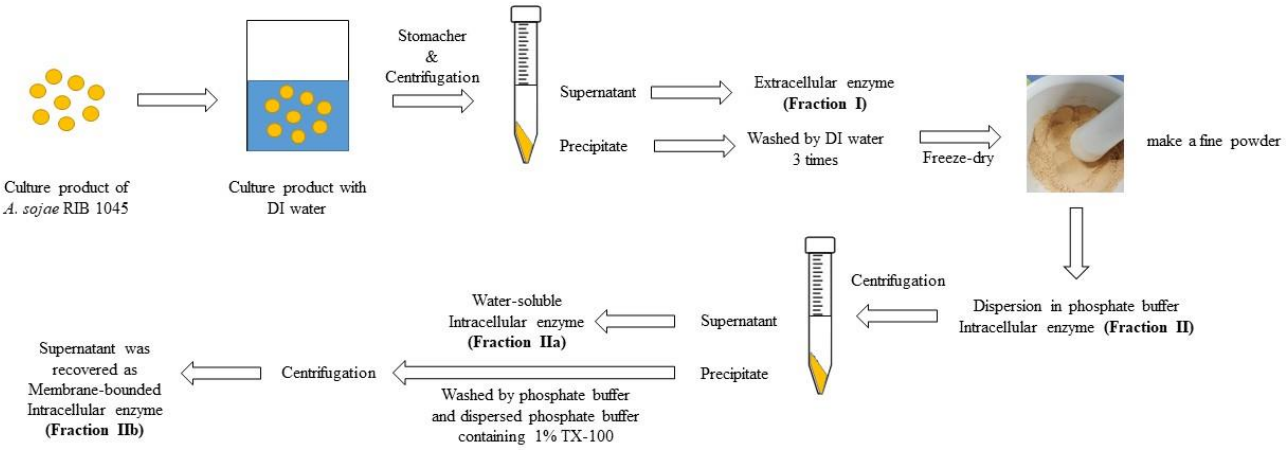
Figure Legends

Figure 1. Flowchart of the fractionation for protease activity measurement

Figure 2. Fluorescent microscope analysis of mycelia of *A. sojae* RIB 1045.
(A) raw culture products; (B) freeze-dried culture products followed by grinding with pestle and mortar.



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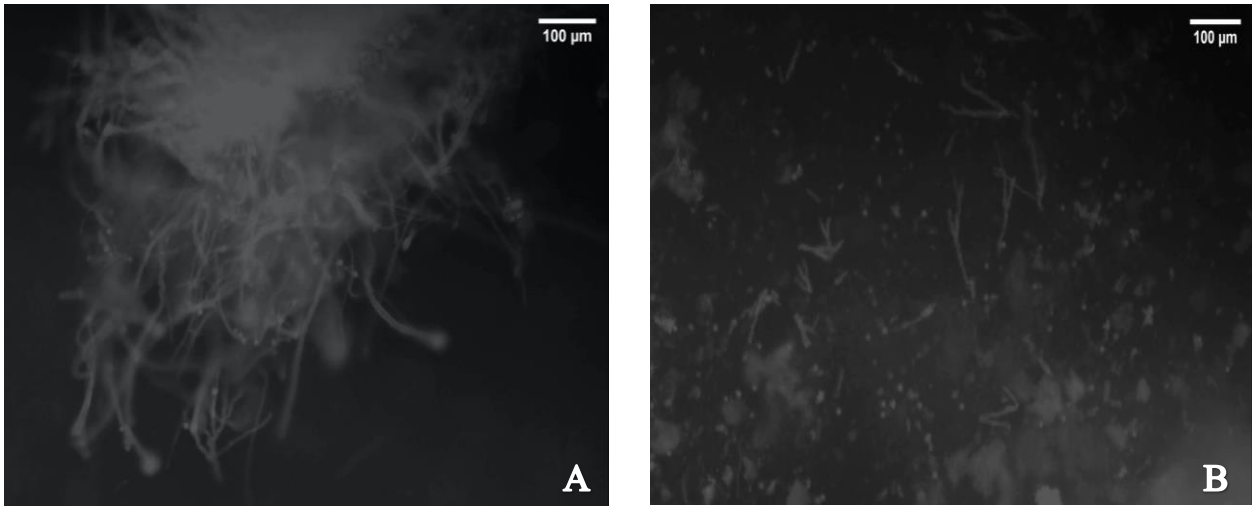

Figure 2. Fluorescent microscope analysis of mycelia of *A. sojae* RIB 1045.

(A) raw culture products; (B) freeze-dried culture products followed by grinding with pestle and mortar.

Table 1. Effect of pH and inhibitors on the protease activity (PU / g of culture)

Sample Fraction	Control	PMSF	Pepstatin A	EDTA	o-phen	E-64
Fraction I						
pH 5.5	2.01 ± 0.02A	1.59 ± 0.07b	0.78 ± 0.03a	1.41 ± 0.19b	1.94 ± 0.00	1.77 ± 0.08
7.0	0.91 ± 0.02B	0.27 ± 0.05a	0.80 ± 0.01	0.75 ± 0.01	0.84 ± 0.05	0.78 ± 0.01
8.5	0.83 ± 0.03B	0.15 ± 0.05a	0.81 ± 0.05	0.84 ± 0.05	0.80 ± 0.10	0.81 ± 0.01
Fraction IIa						
pH 5.5	0.70 ± 0.02A	1.22 ± 0.61	0.24 ± 0.02	0.68 ± 0.04	0.66 ± 0.04	0.71 ± 0.00
7.0	0.19 ± 0.01B	0.08 ± 0.01a	0.17 ± 0.01	0.22 ± 0.02	0.16 ± 0.01	0.19 ± 0.00
8.5	0.20 ± 0.04B	0.06 ± 0.04	0.22 ± 0.01	0.24 ± 0.05	0.31 ± 0.10	0.18 ± 0.02
Fraction IIb						
pH 5.5	0.57 ± 0.03A	0.50 ± 0.05	0.06 ± 0.00a	0.24 ± 0.02b	0.55 ± 0.02	0.56 ± 0.03
7.0	0.26 ± 0.01B	0.21 ± 0.01b	0.07 ± 0.01a	0.19 ± 0.01b	0.26 ± 0.01	0.25 ± 0.00
8.5	0.16 ± 0.00B	0.10 ± 0.01a	0.10 ± 0.00a	0.12 ± 0.01a	0.15 ± 0.00	0.17 ± 0.01

Value are mean ± SE.
a, b indicates a significant difference between the control and the inhibitor's effect to the enzyme fraction under the specific pH condition within the same row ($p < 0.05$).
A, B indicates a significant difference regarding the pH dependence of the control in the individual fraction ($p < 0.05$).

Table 2. The chemical analysis of experimental cheeses

Cheese sample	Water (%)	Protein (%)	Lipid (%)	WSN (%)	FFA (mmol kg^{-1})
Control	37.2 ± 0.1	28.9 ± 0.2	28.4 ± 0.3	13.3 ± 0.4a	100.5 ± 0.4
CP	36.9 ± 0.3a	29.3 ± 0.3	28.8 ± 0.8	20.0 ± 0.4b	110.0 ± 0.3
FDP	37.7 ± 0.2b	28.2 ± 0.2	28.1 ± 0.3	21.1 ± 0.6b	102.0 ± 0.2

Value are mean ± SE.
a, b within a column indicate a significant difference between the samples in each component at $p < 0.05$.