



Title	Adjunctive application of solid-state culture products and its freeze-dried powder from <i>Aspergillus sojae</i> for semi-hard cheese
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1 **Attempt at the adjunctive use of solid-state culture products and its**
2 **freeze-dried powder from *Aspergillus sojae* for semihard cheese**
3 **Short running title: Adjuncts as the raw and freeze-dried culture products of *A.***
4 ***sojae* for cheese**

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22 **Abstract**

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

30 **RESULT:** With different sensitivities to inhibitors, intracellular and extracellular
31 proteases showed the highest activity under the acidic region, which was considered
32 suitable for cheese application. The raw culture product (CP) and its freeze-dried product
33 (FDP) were mixed with cheese curds prepared according to Gouda-type cheese making
34 and were allowed to ripen for three months. Chemical analysis of the products showed
35 13.3% water-soluble nitrogen (WSN) in the control, which had received noncultured
36 media, whereas 20.0% and 21.1% WSN were found in CP and FDP experimental cheese,
37 respectively. Although these adjuncts significantly increased WSN, an insignificant
38 difference was found between CP and FDP. Free fatty acids in all experimental cheeses
39 were similar, showing that CP and FDP caused no rancid defects.

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

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

45 INTRODUCTION

46 *Aspergillus oryzae* and *A. sojae* are pivotal filamentous fungi that have been used in
47 traditional Japanese fermentation products. *A. oryzae* secretes amylases and proteases that
48 contribute to making sake (rice wine), shoyu (soy sauce) and miso (soybean paste). In
49 contrast, *A. sojae* has been used solely for shoyu and miso fermentation because this
50 species possesses high proteolytic activity while having a lower capability when it comes
51 to starch saccharification. In part, this can be attributed to a single copy of the amylase
52 gene, whereas *A. oryzae* possesses three copies of the corresponding genes, though both
53 species are assumed to possess more than 130 proteolytic genes¹.

54 It is well-known that a solid culture is superior to liquid for enzyme production by
55 filamentous fungi. In our previous study², whey protein concentrate (WPC) was used as
56 a solid substrate for *A. oryzae*, and the CPs were mixed with fresh cheese curds prepared
57 under the procedure for traditional semihard type cheese making. In three months of
58 ripening, the water-soluble nitrogen (WSN) in the products increased with the addition
59 of the CPs of *A. oryzae*. This protocol is advantageous because the autoclaving of a WPC
60 solution readily leads to solidification because of the heat denaturation of whey protein.
61 If necessary, the ingredients including other components and/or acidic or alkaline
62 solutions for pH adjustment can be mixed with a WPC solution prior to autoclaving.
63 Furthermore, additional treatments of the CPs that include freeze-dry followed by
64 mechanical particleisation could disrupt the cell walls of filamentous fungi in the
65 substrate. Adjunctive use of the CPs that had received this disruption procedure would
66 allow not only for the action of the extracellular enzymes, but also the intracellular ones
67 during ripening. It may be interesting to apply fungal intracellular enzymes for food
68 development because the traditional Japanese fermentation protocol does not use this
69 method.

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

74 **MATERIALS AND METHODS**

75 *Strain and culture condition*

76 The strain used in the current study was *Aspergillus sojae* RIB 1045, which was kindly
77 gifted by National Research Institute of Brewing (Hiroshima, Japan). This strain was
78 grown on potato dextrose agar (PDA; Merck KGaA) at 30°C for 10 days. Spore
79 suspensions were prepared by adding a 9.0 g/L sodium chloride (NaCl) solution into the
80 grown culture on PDA and then diluted by (9.0 g L⁻¹) NaCl solution to a concentration of
81 2.5×10⁵ spores/mL, which was counted by using haemocytometer (NanoEntek, Korea).

82 Twenty-five grams of WPC80 (Fonterra, New Zealand) and 5 g of glucose was
83 dissolved in 70 g of deionised water and adjusted to pH 4.0 with lactic acid. The solution
84 was divided (10 g) to a 100 mL Erlenmeyer flask and autoclaved at 121°C for 15 min to
85 prepare the solid medium. The medium was inoculated with 150 µL of the spore
86 suspension and cultivated at 20°C for 7 days.

87 *Preparation of the extracellular and intracellular enzymes*

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

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

102 *Fluorecent microscope analysis*

103 The CP and FDP specimens were prepared by placed a solid sample on the glass slide,
104 added one drop of Calcofluor White Stain (Sigma-Aldrich, Canada), and then, one drop
105 of 10% KOH (Kanto chemical, Japan) according to the manufacturer's instructions.
106 Spreading sample and then close with a coverslip and left for 1 min. The physical
107 appearance of the mycelia of CP and FDP were examined by the fluorescence microscope
108 (Olympus BX-50). The Filter (DP50, U-PPMTVC) was used for DAPI excitation (Ex:
109 360-370, Em: 420-460). Photographs were taken by Viewfinder Lite snap camera
110 program (2776 x 2074 pixels) connected to a computer.

111 *Measurement of protease activity*

112 The proteolytic activity was determined as previously described with some
113 modifications³. The substrate, 0.2% casein, obtained by acid precipitation (pH 4.6) from
114 raw skim milk, dissolved in 0.05 M sodium acetate buffer pH 5.5 (700 µL), was incubated
115 with a desired enzyme fraction (50 µL) at 30°C for 2 h. The reaction was terminated by
116 adding 750 µL of trichloroacetic acid (TCA) (Wako, Japan) mixture; containing 0.11 M

117 TCA, 0.22 M sodium acetate and 0.33 M acetic acid, and further incubation was carried
118 out for 15 min. The resulting mixture was centrifuged at 21,130 xg, 25°C for 10 min. The
119 supernatant (1 mL) was recovered and mixed with 2 mL of 0.625 M Na₂CO₃ followed by
120 the addition of 0.7 N Folin reagent (Nacalai Tesque, Kyoto, Japan). The mixture was held
121 at 30°C for 30 min, and its absorbance at 660 nm was determined². The value was
122 corrected by subtracting that obtained from the blank, which was subjected to the same
123 treatments except that a TCA reagent was added to the enzyme prior to the addition of
124 the substrate. Enzyme activity was expressed as micrograms of the released tyrosine per
125 h at 30°C extracted from one gram of the CPs.

126 *Characterisation of the protease activity*

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

132 To observe the effect of pH and inhibitors on the activity, 0.2% casein dissolved in
133 0.05 M buffer including sodium acetate pH 5.5, sodium phosphate pH 7.0 or Tris-HCl
134 pH 8.5 were used in the presence or absence of the following five inhibitors (10 µg/µL
135 Pepstatin A (Peptide institute, Osaka, Japan), 10 mM o-phenanthroline (o-phen)
136 (Fujifilm, Osaka, Japan), 10 mM ethylene diamine tetraacetic acid (EDTA) (Kanto
137 chemical, Tokyo, Japan), 10 mM phenylmethylsulphonyl fluoride (PMSF) (Nakalai
138 Tesque, Kyoto, Japan) and 10 µM E64 (Peptide institute, Osaka, Japan), showing the final
139 concentration during the reaction.

140 ***Preparation of adjunct materials for cheese making***

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

146 ***Cheese making***

147 A batch of cheese making was carried out in June 2019 per the conventional procedure
148 of Gouda-type cheese making. Raw milk was obtained from the experimental farm in the
149 Field Science Center for Northern Biosphere, Hokkaido University. The raw whole milk
150 was standardised with the raw skim milk to adjust its fat content to 3.0% and heated at
151 72°C for 15 sec. After cooling to 31°C, the milk (97 kg) was transferred to a vat, and then,
152 1/1000 volume of 1.5 M CaCl₂ solution and 2% volume of bulk lactic starter (BD culture
153 CH N-01; Chr. Hansen, Denmark) prepared in sterilised skim milk were added. After
154 incubation for 60 min, 4.4 g of calf rennet (Chr. Hansen, Denmark) dissolved in 0.1 M
155 NaCl solution was added to induce coagulation of the milk. After curd formation, it was
156 cut into cubes of 10 mm³ in size and left for 15 min. Subsequently, gentle stirring was
157 performed at 31°C for 30 min, and 30 kg of whey was discarded. Then, 24 kg of warm
158 water (60°C) was added to reach 39°C, followed by 15 min of agitation at this
159 temperature. After the drainage, the curds were recovered and weighed to be mixed with
160 1% weight of the CP, 0.26% weight of the FDP or 1% weight of the uninoculated WPC
161 solid culture (control) by hand uniformly to transferred to 1 kg size of mould for Gouda
162 type cheese. These three types of cheeses were prepared in triplicate. Following brief
163 pressure (1.0 kg/cm² for 10 min), inversion and the second stage of pressure (1.5 kg/cm²

164 for 50 min) were performed. The curds were cooled in water overnight and treated with
165 dry salting (20 g of NaCl per 1 kg cheese). The curds were matured at $11.5 \pm 1^\circ\text{C}$ for 12
166 weeks with a relative humidity of 85 - 90%. After 15 days of the manufacture, the cheese
167 surface was coated with wax (Paramelt, Netherland) and left for ripening.

168 *Chemical analysis of cheese*

169 All assays were performed in triplicate.

170 The moisture was determined according to the International Dairy Federation
171 (IDF)'s recommendation⁴. Fat and protein were measured using the Association of
172 Official Agricultural Chemists (AOAC) method^{5,6}.

173 WSN was determined according to the method of Kuchroo and Fox⁷, with some
174 modifications: a sample (5 g) was added to 25 mL of deionised water and, after treatment
175 with a stomacher for 5 min, maintained at 40°C for 1 hr. The sample was centrifuged, and
176 the supernatant was passed through glass wool to entrap lipids. The resulting filtrate was
177 subjected to the micro Kjeldahl method⁵.

178 After the extraction of the fat according to the AOAC protocol⁶, free fatty acids
179 (FFAs) were extracted by the phenol-red method⁸. Oleic acid was used as the standard
180 and converted to the content of oleic acid (mmol) in 1 kg of cheese.

181 *Statistical analysis*

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

188 **RESULTS**

189 *Effect of freeze-dry treatment on the CPs*

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

195 *Protease Activity*

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

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

210 *Cheese Composition*

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

217 **DISCUSSION**

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

227 All fractions contained several protease species with different rational profiles, as
228 shown in Table 1. Serine protease, which is sensitive toward PMSF⁹, is likely to be
229 involved, as confirmed in *A. oryzae*¹⁰, *A. sojae*¹¹ and many kinds of *Aspergillus* strains¹².
230 As the acidic serine protease from *Aspergillus* sp., *A. oryzae* and *A. sojae*, aorsin is well-
231 known¹³. Although pepstatin A inhibited protease activity¹⁴ in fraction IIb in every pH
232 condition, the inhibition was exclusive under the acidic condition in fraction I. Although
233 aspartic protease of aspergillopepsin A (pepA)¹⁵ or aspergillopepsin O (PEPO)¹⁶ has been

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

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

258 Although no statistical difference of WSN was found in the cheese using CP and
259 FDP, the addition of those preparations led to 20.0 – 21.1% of WSN, which was higher
260 than that of the control (13.3%) which represented the typical gouda-type cheese^{2,18}. In
261 our previous study², maximum WSN increase was recorded using the CPs of *A. oryzae*
262 AHU 7139, whose WSN and its control was 14.7% and 12.5%, respectively. Furthermore,
263 some of the resulting cheese products using CPs from *A. oryzae* AHU 7139 and *A. oryzae*
264 AHU 7146 showed a remarkable increase of FFA compared with the control cheese,
265 whereas no increase of FFA was found when *A. sojae* was used in the current study. In
266 fact, we recognised neither bitter tastes nor a rancid flavour because of bitter peptide or
267 volatile FFAs; however, we focused on chemical analysis rather than organoleptic
268 assessment. These results suggest that application of *A. sojae* RIB 1045 into cheese is
269 unlikely to cause the defects related to rancidness, and *A. sojae* would be more
270 advantageous than *A. oryzae* here. In addition, our previous protocol introduced a filter
271 on the solid whey substrate to obtain spore-free CPs through the removal of hyphae on
272 the filter. However, we recognised no fungal growth during ripening in the current study
273 despite using raw CPs with aerial hyphae. Thus, we can conclude that this separation
274 procedure of aerial hyphae using the filter on the solid culture can be neglected.

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

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

284 **ACKNOWLEDGEMENTS**

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286 Northern Biosphere, Hokkaido University, for their assistance in cheese manufacturing.

287 **CONFLICT OF INTEREST**

288 The authors declare that they have no conflict of interest.

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339 **Figure Legends**

340 **Figure 1.** Flowchart of the fractionation for protease activity measurement

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

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

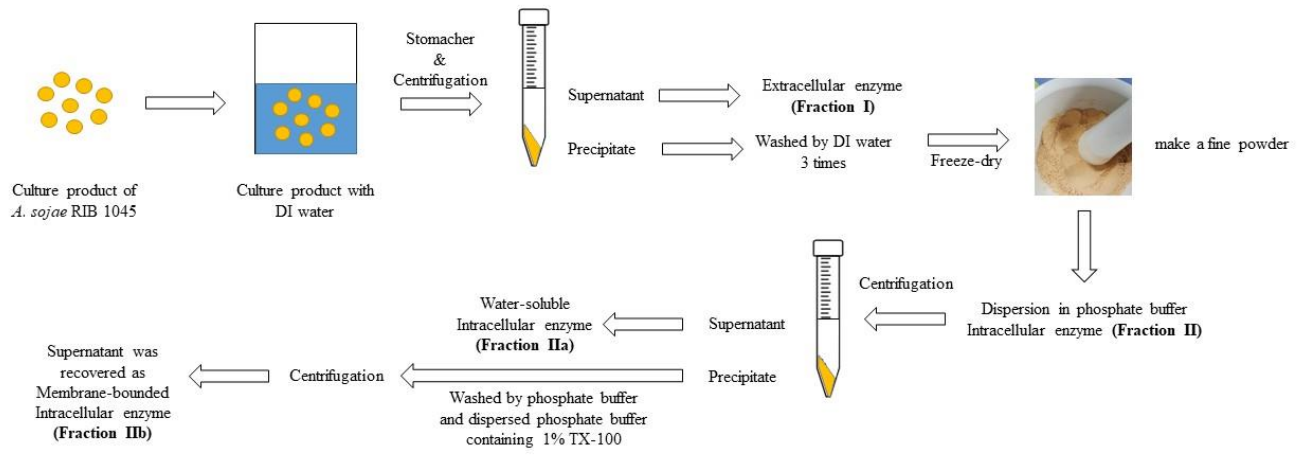


Figure 1. Flowchart of the fractionation for protease activity measurement

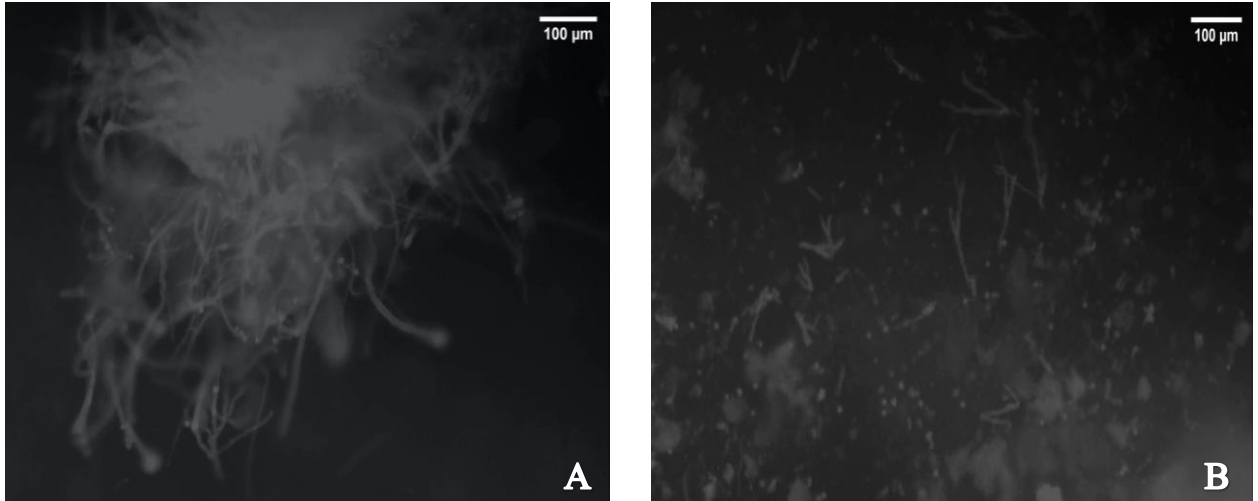


Figure 2. Fluorescent microscope analysis of mycelia of *A. sojae* RIB 1045.

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

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.