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Physicochemical Properties and Fibrinolytic Activity of Ginseng Powder Fermented with *Bacillus subtilis* Isolated from *Cheonggukjang*

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Abstract

In order to obtain all the properties of fermented ginseng, we fermented ginseng using *Bacillus subtilis (B. subtilis)* isolated from *Cheonggukjang*. A sterilized ginseng medium was made with 4-year-old ginseng powder and distilled water (300% ginseng powder [w/w]), and the ginseng was fermented by *B. subtilis* (1% ginseng medium) followed by incubation at 37˚C for 3, 5, 7, and 10 days. The growth of *B. subtilis* in the ginseng medium significantly increased up to 9 log CFU/g, but no significant difference was observed after 3 days. As the fermentation progressed, the ginsenoside Rd and Rg+Rh1 contents increased by 255.3% - 322.5% and 165.6% - 228.6%, respectively, whereas the Rc, Re, and Rg1 contents decreased by 30.7% - 39.6%, 10.5% - 12.8%, and 16.2% - 16.6%, respectively. After 3 days of fermentation, a 6.25% - 7.12% viscous substance was produced; thereafter, the viscous substance was gradually reduced until it disappeared. The viscosity of the medium significantly decreased with a longer fermentation time. Fibrinolytic activity increased during 3 - 10 days of fermentation, indicating a relative activity of 85.0% - 100.0%.

Keywords

Bacillus subtilis, Fermentation, Fibrinolytic activity, Ginseng, Ginsenoside

1. Introduction

As a perennial semi-shade plant belonging to the genus *Panax* of the family Araliaceae and a representative product of Korea, ginseng (*Panax ginseng* C. A. Meyer) has been widely used as medicinal herb in folk medicine and oriental medicine for thousands of years [1] [2]. The pharmacological components of

ginseng include saponins, polyacetylenes, polyphenolic compounds, and acidic polysaccharides [3]. Among these, saponin is known as a major substance with the highest pharmacological efficacy, and more than thirty types have been isolated and named ginsenosides with known chemical structures [4]. Various benefits of ginseng have been reported, including anti-cancer effect, anti-oxidative effect, arteriosclerosis prevention, hypertension prevention, hepatic function improvement, anti-hangover, anti-fatigue, anti-stress, anti-aging, brain function improvement, and anti-inflammatory activity. In addition, studies on the conversion of ginsenosides with excellent efficacy by various treatments, including acid, heat, and microorganisms, have been actively conducted [5] [6]. Ginsenoside conversion in ginseng by microorganisms is well known [7] [8] [9] [10]. Ginseng is made of 3% - 6% saponin, 12% - 16% nitrous compounds, 1% - 2% fat-soluble compounds, 0.05% vitamins, 60% - 70% carbohydrates, and 4% - 6% inorganic compounds; such a composition is suitable for the components of the medium in fermentation or bioconversion [11]. The fermentation of ginseng using microorganisms can provide useful live microorganisms as probiotics as well as saponins with improved effectiveness through the conversion of the ginsenoside structure by decomposing the sugar part of the ginsenoside with a glycoside structure [12]. Currently, commercially used probiotics include *Lactobacillus*, *Lactococcus*, *Bifidobacteria*, *Bacillus subtilis (B. subtilis)*, *Aspergillus bacteria*, and *Butyric-acid bacteria* [11]; in particular, the viscous substance obtained from fermented metabolites of *B. subtilis* is composed of glutamic acid polymerized to γ-glutamic acid (PGA) and fructose polymerized to fructan-type levan [13]. These viscous substances have important influences on the quality-related characteristics of fermented products. Specifically, PGA, a kind of microorganic, high-molecular substance, shows physiologically active functions such as an immune enhancing effect and an anti-cancer effect, and is popular as a functional material for foodstuffs, pharmaceuticals, and even cosmetics due to its excellent moisturizing property [14]. *B. subtilis* isolated from *Cheonggukjang* has been reported as an excellent strain that produces poly-γ-glutamic acid (PGA), a physiologically active viscous substance, and fibrinolytic enzymes [15]. In general, when red ginseng marc [16], defatted soybean [17], soybean curd residue [18], and ginseng [12] [19] are fermented using *B. subtilis*, fermentation is carried out at 37˚C - 42˚C for 24 - 72 hours, but studies of fermentation for over 72 hours are seldom conducted. As such, this study was performed to confirm the changes of *B. subtilis* and ginsenosides according to the fermentation time-by fermenting a ginseng medium inoculated by *B. subtilis* for up to 240 hours-and to evaluate the physical properties and fibrinolytic activity following fermentation, in order to provide optimal fermentation conditions suitable for the production of fermented ginseng solids with improved functional components.

2. Materials and Methods

2.1. Materials

Ginseng powder was made with four-year-old root ginseng grown in *Geumsan*.

Cheonggukjang was purchased as traditional *Cheonggukjang* manufactured in *Cheongyang, Chungnam*.

2.2. Pure Culture Isolation

To isolate a pure culture of *B. subtilis* from the *Cheonggukjang* manufactured in Cheongyang, Chungnam, cell subculture was performed in a nutrient broth until a single colony was formed. 1 uL of an isolated single colony was collected using a loop and inoculated onto a slide, and then 1 uL of α-cyano-4-hydroxycinnamic acid (CHCA) Matrix solution was dropped onto the colony. The microorganism was identified by placing the slide in VITEK MS (bioMériux, Australia) Matrix-Assisted Laser Desorption/Ionization Time-of Flight Mass Spectrometry (MALDI-TOF). Escherichia coli (E. Coli) American Type Culture collection (ATCC) 8739 was used as the quality assurance (QA) strain. Based on the first identification results, the microorganism presumed to be *B. subtilis* was sent to BIOFACT Co., Ltd. and was confirmed as two types of *B. subtilis* of *Cheonggukjang* origin through 16S rRNA analysis.

2.3. Ginseng Fermentation

The ginseng powder (25 g) obtained from 4-year-old root ginseng cultivated in Geumsan was mixed with distilled water (75 g) 3 times its weight in a 500 mL bottle and sterilized at 121˚C for 15 minutes to prepare the ginseng solid medium. Onto the medium, 1% (w/w) each of the two types of *B. subtilis* purely isolated from *Cheonggukjang* was inoculated and cultured in an incubator at 37˚C for up to 10 days.

2.4. Bacterial Count

To measure the bacterial count of *B. subtilis* isolated from *Cheonggukjang*, the ginseng fermentation culture medium was diluted in peptone water and smeared on the nutrient agar, and then cultured in an incubator at 37˚C for 24 hours.

2.5. Ginsenoside Content

The ginsenosides were analyzed by modifying the HPLC analysis method proposed by In *et al.* [20]. After freeze-drying the fermented ginseng solids, a 0.5 g sample was collected in a 15 mL tube, to which 10 mL of 70% MeOH was added and stirred, and then treated by ultrasound for 30 minutes. It was then centrifuged at 3000 rpm for 10 minutes, and the supernatant was filtered through a 0.45 um PTFE filter and used in the high-performance liquid chromatography (HPLC) analysis. The measurements were taken using the HPLC system (Chrompass Data System, JASCO, Japan) at 203 nm, with an auto sampler (AS-2051 plus, JASCO), Chrompass software (ver. 1.8.6.1, JASCO), and a photodiode array detector (MD-2010, JASCO). The analytical column used was a 5 μm Symmetry C18 column (250 × 4.6 mm, SunFireTM, Waters Corp, USA); the injection was 20 uL, the flow rate was 1.6 mL/min, and the column temperature was 35˚C. The mobile phase employed was (A) water and (B) acetonitrile

deaerated by an ultrasonic cleaner, and the gradient system was established as shown in Table 1. Standard ginsenoside materials including Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Re, Rg1, Rg2 + Rh1 and Rh1(r) were purchased from Fleton Natural Products Co., Ltd (Chengdu, China).

2.6. Viscous Substance Content

5 g of the ginseng medium was mixed with the same amount of distilled water and homogenized at 25˚C for 30 minutes, and then centrifuged at 3000 rpm for 15 minutes; 5 mL of the supernatant was then obtained. Afterward, it was evaporated and dried at 105˚C, and the weight was measured and expressed as the dry weight of the sample.

2.7. Viscosity

The viscosity of the medium was measured at 25˚C using a rheometer (Ar2000, TA instruments, New Castle, DE, USA). The shear rate showed 100 points in the range of 0 - 100 1/s.

2.8. Fibrinolytic Activity

The fibrinolytic activity of the ginseng solid medium was measured using the method proposed by Astrup and Mullertz [21], though with some modifications. Fibrinogen was completely dissolved in a 50mM phosphorus buffer solution (pH 7.4, 0.15M NaCl included) to a final concentration of 0.3%; and then 5 mL of this fibrinogen solution and the same volume of 2% agarose (sterilized agar solution) were mixed. 500 uL of thrombin (100 NIH unit/mL) was added to the aforesaid mixed solution and fully mixed with it, and then the solution was immediately poured onto a petri-dish and left to stand at room temperature for 30 - 60 minutes for solidification to obtain the fibrin plate. To measure the activity, 7 holes with a diameter of 5 mm were made on the fibrin plate using a pasteur pipette, and then 4 mL of distilled water per 1 g of medium was added and mixed for 30 minutes and centrifuged; 20 uL of the supernatant was injected into the mixture and reacted at 37˚C for 5 hours, and then the area of the transparent

Table 1. Composition of the mobile phase employed in the gradient HPLC system.

Time (min)	Composition of mobile phase (%)	
	Water	Acetonitrile
0	80	20
5	80	20
25	72	28
35	67	33
40	59	41
45	20	80
47	20	80
50	80	20

circle thus generated was calculated. For the control plot, a purified fibrinolytic enzyme, plasmin (1.0 unit/mL), was used. The fibrinolytic activity of the extract was calculated by converting the relative ratio of the fibrinolytic area of the sample into that of the control plot.

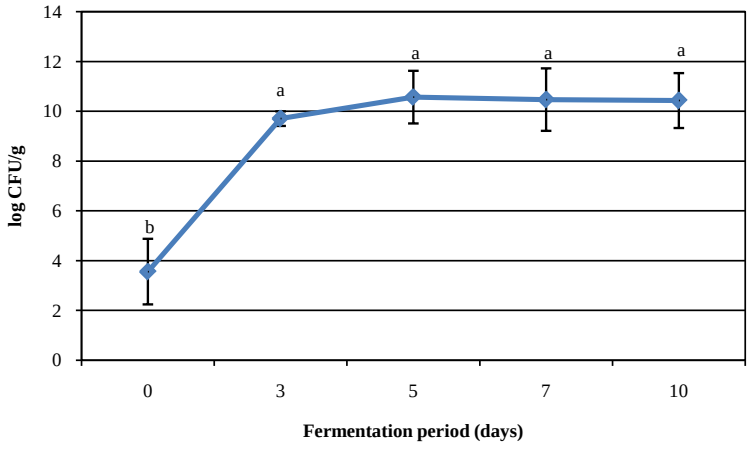
2.9. Statistical Analysis

Statistical analysis was performed using SAS software (Ver. 9.0, Cary, NC, USA). The significance of the results of the study was tested using Duncan's multiple-range test at the 5% level.

3. Results and Discussion

3.1. Bacterial Count

The bacterial count of *B. subtilis* during fermentation was measured as shown in Figure 1. The counts of the A and B strains immediately after inoculation were 3.56 and 3.74 log CFU/g, respectively, but they proliferated to over 9.00 log CFU/g after 3 days of fermentation, regardless of the kind of strain. Later, their counts were 10.47 log CFU/g and 10.25 log CFU/g, respectively, at up to 7 days



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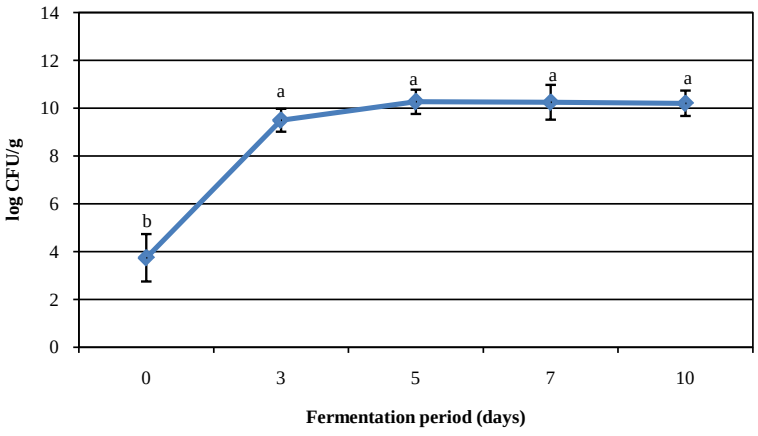


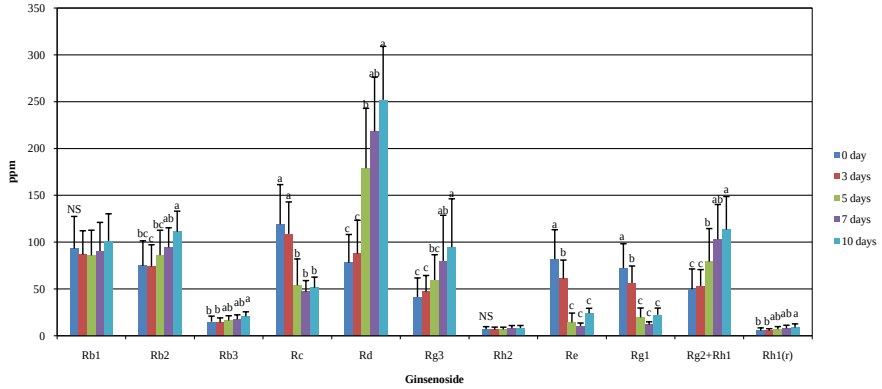
(b)

Figure 1. Growth of *B. subtilis* during ginseng fermentation.

of fermentation—with a slightly higher count in the A strain but decreased slightly to 10.43 log CFU/g and 10.20 log CFU/g, respectively, after 10 days of fermentation without any significant difference. In their study involving screening for ginseng-fermented microorganisms, Kim *et al.* [12] reported that, when *Bacillus* was inoculated onto a 2.5% ginseng powder medium and cultured, the growth was more than 6 log CFU/mL for 24 hours, gradually increasing to 7 log CFU/mL or showing quiescence. The proliferation of the strain was considered sufficient because there was no significant difference in the count after 3 days of fermentation, regardless of the type of strain.

3.2. Ginsenosides

The changes in the ginsenoside content during fermentation are shown in **Figure 2**. Ginsenoside analysis was performed on 11 kinds of ginsenosides including Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Re, Rg1, Rg2+Rh1, and Rh1(r). When the A strain was inoculated and fermented in the ginseng medium, the ginsenosides Rb2, Rb3, Rd, Rg3, Rg2 + Rh1, and Rh1(r) significantly increased by 48.68%, 37.84%, 222.71%, 129.17%, 128.44%, and 59.78%, respectively, while Rc, Re, and Rg1 decreased by 60.40%, 87.16%, and 59.78%, respectively, compared to the initial amounts. In the case of the B strain, Rb1, Rc, Re, and Rg1 decreased by 26.89%, 69.33%, 89.52%, and 83.74%, respectively, whereas Rd and Rg2 + Rh1



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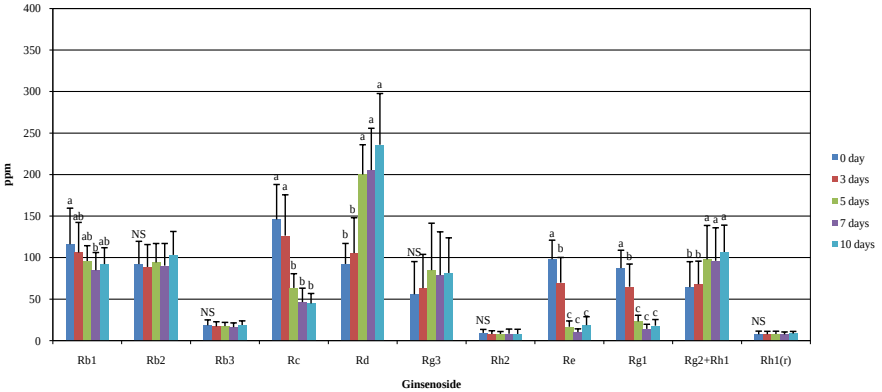


(b)

Figure 2. Ginsenoside contents during ginseng fermentation.

increased by 155.40% and 65.61%, respectively, as the fermentation time increased. It was confirmed that the decrease in Rc, Re, and Rg1 and the increase Rd and Rg2 + Rh1 are common changes during fermentation, regardless of the strain type. These results are similar to those reported in the studies on ginseng fermentation using *B. subtilis* by Kim *et al.* [12] and Lim *et al.* [19]. In particular, Kim *et al.* [12] confirmed that Rb1 and Rc decreased and Rd increased in ginseng fermentation using *B. subtilis*. Lim *et al.* [19] also reported that Rb1 and Rc decreased from 4.93 and 3.03 mg/g to 2.46 and 2.62 mg/g, respectively, whereas Rd increased from 0.66 to 7.21 mg/g. Kim *et al.* [12] suspected that Rc is degraded by ginsenoside-α-arabinofuranase, and that the sugar part of Rb1 is degraded to Rd by *γ*-glucosidase. Park *et al.* [22] and Zhang *et al.* [23] also reported that Rc is considered to be converted into Rd by ginsenoside-*α*-arabinofuranase and Rb1 into Rd by *γ*-glucosidase. These results can be said to confirm the potential of probiotics when eaten raw, and both nutritional and functional benefits are expected depending on the conversion of ginsenosides.

3.3. Viscous Substance Content

The major component of the viscous substance in *Cheonggukjang* is *γ*-polyglutamate, one of the metabolites produced by microorganisms belonging to the genus *Bacillus* during fermentation [24] [25] and a biopolymer with functionality such as osteoporosis prevention [26]. Thus, the amount of viscous substances produced during ginseng fermentation using the strain of *Cheonggukjang* origin was confirmed, as shown in **Figure 3**. The amount of viscous substances observed in the A strain medium and the B strain medium after 3 days of fermentation was 6.253% and 7.120%, respectively, decreasing to 2.773% and 3.553%, respectively, after 5 days of fermentation. However, after 7 days of fermentation, no viscous substances were observed in the medium. Polyglutamates, the major viscous substance in *Cheonggukjang*, generally occur as 2.15% - 6.03% [17]. However, that the viscous substance content of fermented ginseng in the study was a maximum of 7.120%, which was 1.18 - 3.31 times higher than in *Cheong-*

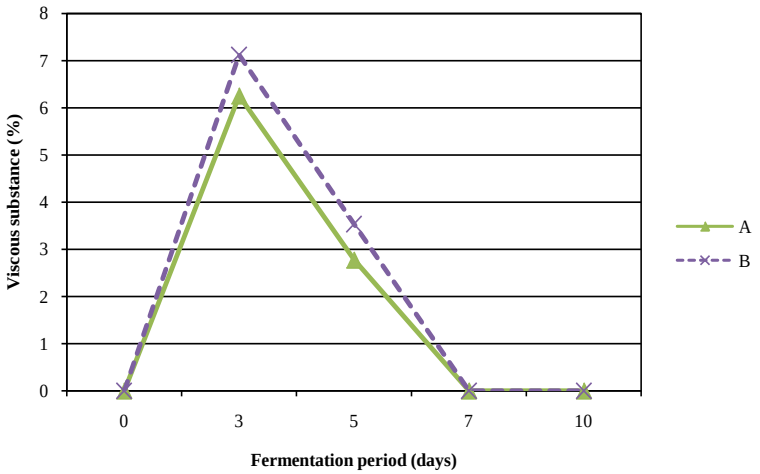
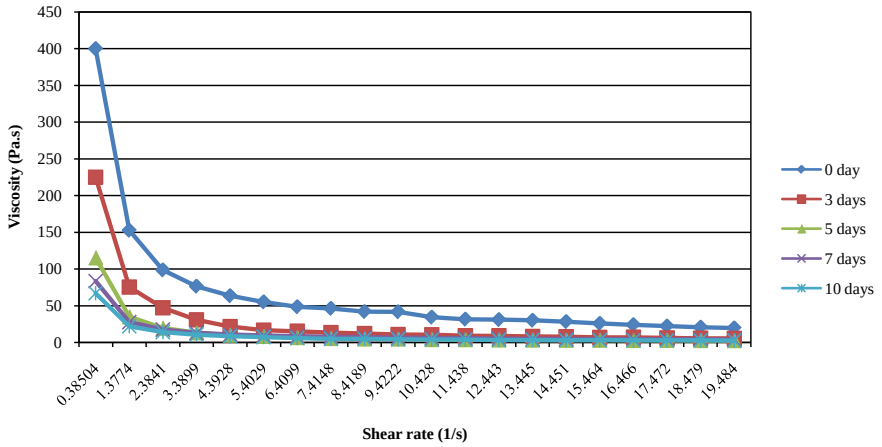


Figure 3. Viscous substance production during ginseng fermentation.

gukjang. When the amount of viscous substances was compared, the content from the B strain fermented medium was 1.14 - 1.27 times higher than that from the A strain, suggesting a more active production of viscous substances from the medium using the B strain. Thus, it is believed that the functionality of viscous substances will be utilized more when fermented ginseng is used.

3.4. Viscosity

The changes in the physical properties of the ginseng medium during fermentation showed that the viscosity gradually decreased as the shear rate decreased, and then stabilized to a certain level of viscosity regardless of the type of strain (Figure 4). The viscosity of the medium fermented using the A strain was initially 399.85 Pa.s, but this gradually decreased to 224.9 Pa.s, 115.3 Pa.s, 83.4 Pa.s, and 66.9 Pa.s as fermentation progressed. In the case of the B strain, the viscosity was 362.6 Pa.s immediately after inoculation and 258.5 Pa.s after 3 days of fermentation, 96.8 Pa.s after 5 days of fermentation, 75.8 Pa.s after 7 days of fermentation, and 78.01 Pa.s after 10 days of fermentation, showing that the initial viscosity of the medium inoculated by the B strain also decreased as fermen-



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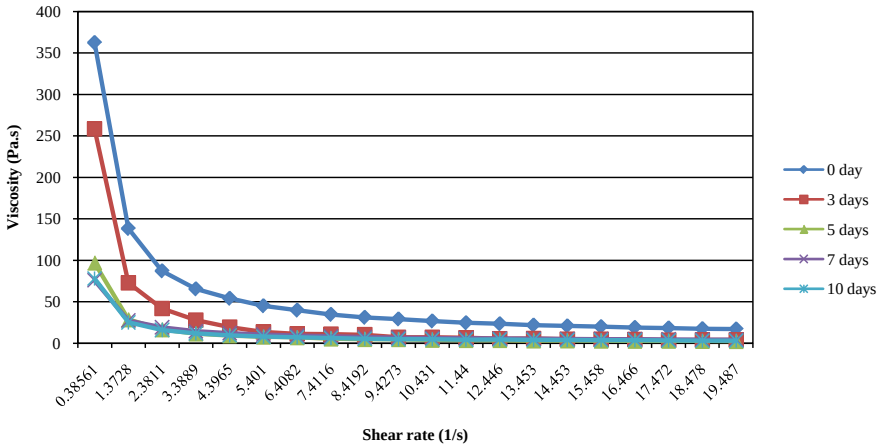

(b)

Figure 4. Viscosity of ginseng medium during ginseng fermentation.

tation progressed. Initially, the viscosity was high due to the viscosity of the ginseng solid medium itself, but it gradually decreased by fermentation and then stabilized at higher shear rates as the fermentation period increased. When comparing the A and B strains, the initial viscosity immediately after the *Bacillus* inoculation was higher in the medium inoculated by the A strain; after 3 days of fermentation, however, the initial viscosity of the B strain was 33.6 Pa.s higher compared to the A strain, meaning that the viscous substance content and the viscosity were proportional, which is thought to be a result of the production of viscous substances with polymerized fructose and glutamic acid by *B. subtilis* [27]. Lee *et al.* [28] manufactured *Cheonggukjang* by controlling the ratio of *B. subtilis* and *Lactobacillus sakei* with an inoculation concentration of 1% and reported that the viscosity was higher with a higher ratio of *B. subtilis* MC31. This latter finding is consistent with the result that the viscosity increased with an increasing amount of viscous substances. Note, however, that the decrease of viscosity after 3 days of fermentation quickly occurred in the fermented ginseng including the B strain. This result suggests that poly *γ*-glutamate depolymerase, a degrading enzyme of viscous substances, is generated as an extracellular enzyme, degrades viscous substances, and decreases the viscosity [29].

3.5. Fibrinolytic Activity

Strains in the genus *Bacillus* used in the production of *Cheonggukjang* generally have fibrinolytic activity, but some strains without fibrinolytic activity actually exist. Therefore, the fibrinolytic activity of fermented ginseng by *B. subtilis* isolated from *Cheonggukjang* was identified (**Figure 5**). The fibrinolytic activity of the medium was not confirmed immediately after inoculation regardless of the strain, but the activity of the medium fermented by the A strain was 85.0% after 3 days of fermentation. Moreover, fibrinolytic activity was similar or increasing as fermentation continued. The fibrinolytic activity of the ginseng medium fermented by the B strain was 100.0% after 3 days of fermentation-which was the same effect as plasmin 1.0 unit/mL-and 97.5% after 5 days of fermentation, which was 2.5% lower compared to the 3-day fermentation, but fibrinolytic activity was maintained for up to 10 days of fermentation, demonstrating a 10.0% - 15.0% higher effect than that using the A strain. This result is consistent with the one reported by Mann *et al.* [30] wherein fibrinolytic activity was observed in *Cheonggukjang* fermented for 72 hours using *B. subtilis* MC 31. Moreover, No *et al.* [31] *Doenjang* made with *Aspergillus oryzae* D-2 showed 5 mm of fibrinolytic activity occurring immediately after its production showed an increase in fibrinolytic activity of up to 20 mm until day 30 of fermentation, followed by a gradual decrease after 30 days. Thus, it is expected that the fibrinolytic activity will not decrease significantly until 10 days of ginseng fermentation, but will decrease as the fermentation period is extended.

4. Conclusion

Fermented ginseng converted structure of ginsenoside have an advantage about

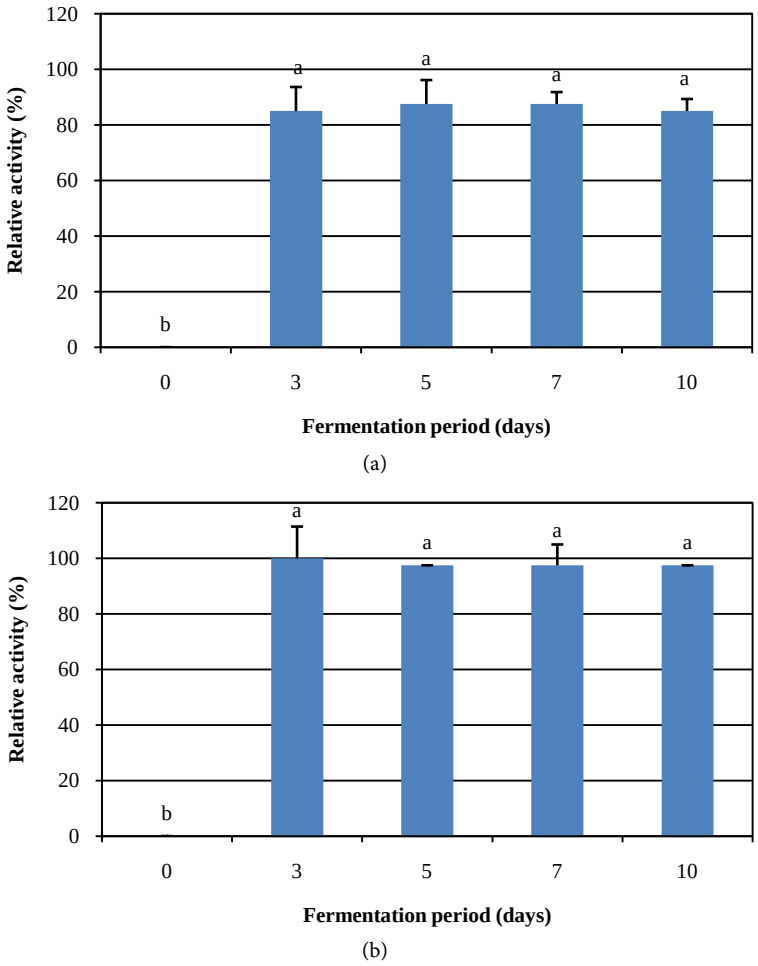

Figure 5. Fibrinolytic activity during ginseng fermentation

eating high-effective saponin than general raw ginseng. Generally, *Bacillus subtilis* can produce the extracellular viscous materials containing polyglutamic acid and fructan which provide a consistency and taste. Viscous substance made by *B. subtilis* fermentation has been known to effects on antimicrobial, antihypertensive and immunostimulating activity. In order to obtain all the properties of fermented ginseng and viscous substance, we fermented ginseng using *B. subtilis* isolated form *Chenggukjang* for 10 days. As the fermentation progresses, growth of *B. subtilis* in ginseng media was increased to 9 log CFU/mL after 72 hour fermentation. The number of *B. subtilis* was increased more than 10 log CFU/mL but the fermentation was completed because no difference was observed. Ginsenoside Rd and Rg2 + Rh1 were increased after fermentation. In contrast, Rc, Re and Rg1 contents were decreased. Especially, Rd content changed to the greatest extent. After 3 days, viscous substance was produced 6.25% (A), 7.12% (B) and gradually reduced to disappeared. The longer the fermentation time, viscosity of medium was significantly decreased. The production of fibrinolytic activity was increased fermentation after 72 hours, indicating the relative activity of 85.00% (A), 100.00% (B), respectively. Moreover, these effects lasted until 10 days. Overall, 5 days was an appropriate period to use viscous substance, high-

effective saponin and great fibrinolytic activity of fermented ginseng by *B. subtilis*.

Acknowledgements

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