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RESEARCH NOTE

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Distribution of poly-γ-glutamate (γ-PGA) producers in Korean fermented foods, *Cheongkukjang*, *Doenjang*, and *Kochujang*

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Abstract Poly-γ-glutamate (γ-PGA) has great potential as a biodegradable polymer in a broad range of industrial fields such as food, cosmetics, medicine and water treatment. In order to isolate γ-PGA producers that are suitable for specific industrial applications, 653 *Bacillus*-like strains were isolated from 439 varieties of three Korean fermented foods, *Cheongkukjang, Doenjang*, and *Kochujang*, which were collected from different regions across Korea. A very high level of γ-PGA production was demonstrated in 4.7%, 1.8%, and 3.0% of the *Bacillus*-like strains isolated from *Cheongkukjang, Doenjang*, and *Kochujang* samples, respectively, which produced a viscous substance to such extent that it overflowed to the lid of the plate on the glutamate-dependent γ-PGA production plates. On glutamate-independent γ-PGA production plates, 5.1%, 5.9%, and 6.1% of *Bacillus*-like strains isolated from *Cheongkukjang, Doenjang*, and *Kochujang* samples, respectively, showed high production. The maximum γ-PGA production yields were 32.5 g/L and 5 g/L, depending on the purification methods in the glutamate-dependent media, with the higher yield resulting from a simple precipitation of γ-PGA by either methanol or ethanol and dialysis. The viscous substance produced by each strain showed different morphological characteristics, suggesting that isolated γ-PGA producers could produce various types of γ-PGA.

Keywords: poly-γ-glutamate (γ-PGA), *Bacillus* sp., *Cheongkukjang, Doenjang, Kochujang*

Introduction

Poly-γ–glutamate (γ-PGA) is an anionic homo-polyamide in which D- and L-glutamate units are connected by amide linkages between α-amino and γ-carboxylic groups. It is water soluble, biodegradable, edible, and non-toxic toward humans and the environment. Such unique characteristics have attracted considerable interest from a broad range of industries. The potential applications of γ-PGA and its derivatives are very diverse. For example, it is used as thickeners, humectants, bitterness relieving agents, cryoprotectants, sustained release materials, drug carriers, curable biological adhesives, biodegradable fibers, highly water absorbable hydrogels, biopolymer flocculants, heavy metal- and radionuclide-absorbers, animal feed additives, osteoporosis-preventing factors, gene vectors, dispersants, and enzyme-immobilizing materials [reviewed by Shih and Van (1) and Shih *et al.* (2-3)].

For industrial applications of this promising biopolymer, it needs to be produced at an economical price and have a structural diversity with different physicochemical characteristics. Therefore, it is very important to obtain microorganisms that can produce γ-PGA abundantly in cheap media and with diverse structures such as different polymer length and D-/L-glutamate ratio. Since it was first discovered as a capsule of *Bacillus anthracis*, several *Bacillus* species have been shown to produce γ-PGA [reviewed by Shih and Van (1), Kunioka (4), and Ashiuchi and Misono (5)]. *B. subtilis* (*Chungkookjang*) has been isolated to produce a high molecular weight γ-PGA (>1,000 kDa) from the Korean fermented food *Cheongkukjang* (6). Because the high molecular weight γ-PGA has advantages in certain applications, this microorganism is more suitable than *B. subtilis* (*natto*) that produces γ-PGA with variable molecular weight ranging from 10 to 1,000 kDa (7-8). *Cheongkukjang* is a traditional Korean fermented-soybean sauce which contains the same viscous substance as Natto. The main fermenting microorganism of *Cheongkukjang* is *Bacillus subtilis*. There are many kinds of *Cheongkukjang*s, each with its own unique taste and flavor, which are affected by several factors including fermentation methods and soybean cultivars (9, 10). We thought that the fermenting microorganisms were also important factors to confer the unique flavor. Therefore, we reasoned that regionally different *Cheongkukjang*s might be fermented by different *Bacillus* species that produce different types of γ-PGA with different physicochemical characteristics. In addition, other fermented foods such as *Doenjang* and *Kochujang* should contain *Bacillus* sp. that produces γ-PGA. In this study, many varieties of the three Korean fermented foods, *Cheongkukjang*, *Doenjang*, and *Kochujang*, were collected from different regions of Korea to investigate and isolate γ-PGA producers with more advantageous properties for industrial applications. The isolated microorganisms were grouped into classes based on the quantity and types of γ-PGA produced.

Materials and Methods

Materials and reagents The *Cheongkukjang, Doenjang*, and *Kochujang* samples collected from all over the nation were mainly homemade or regional varieties (Fig. 1). All chemicals used were of reagent grade.

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Isolation of *Bacillus*-like microorganisms Each food sample (5 g) was dissolved in 25 mL of peptone water (0.1 % peptone). After serial dilution, samples were spread onto NA plates (0.3% beef extract, 0.5% pancreatic digest of gelatin, 1.5% agar) followed by incubation at 37°C for 16 hr. Colonies showing the morphology of *Bacillus* were isolated separately and cultured purely. Morphological and biological characteristics of some isolates were investigated according to the standard procedures (11-13). General and biochemical characteristics were examined by an API 50 CHB kit (bioMerieux Vitek, Inc., USA).

Qualitative evaluation of the production of the viscous substance The isolated strains were streaked onto agar plates containing glutamate [8% sucrose, 2% L-glutamate, 0.5% $Na_2HPO_4 \cdot 2H_2O$, 0.15% $MgSO_4 \cdot 2H_2O$, 0.015% biotin, 1.5% agar (14)], for evaluation of glutamate-dependent γ-PGA production, and on non-glutamate containing plates [2% glucose, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.015% $CaCl_2 \cdot 2H_2O$, 0.015% $MnSO_4 \cdot 4H_2O$, 0.015% $ZnCl_2$, 0.4% NH_4Cl, 1.5 % agar (15)], for glutamate-independent γ-PGA production. The plates were incubated at 37°C and γ-PGA production was evaluated qualitatively by eye based on the visible amount every 24 hr for 3 days.

Purification and quantitative evaluation of γ-PGA A strain was cultured in 50 mL of γ-PGA production media [2% glutamate, 5% sucrose, 2% $(NH_4)_2SO_4$, 0.27% KH_2PO_4, 0.42% Na_2HPO_4, 0.05% NaCl, 0.5% $MgSO_4$, MS vitamin solution (6, 16)]. For preparation of crude γ-PGA, culture broth was precipitated using methanol and the precipitate was freeze-dried after washing with H_2O, as described by Goto and Kunioka (17). Pure γ-PGA was obtained from culture broth by the method of Ashiuchi *et al.* (18). This method applies sulfuric acid and proteinase K treatment to remove polysaccharides and γ-polypeptides. The final γ-PGA solution was dialyzed three times against 2 L of distilled H_2O at 25°C overnight. The solution was lyophilized, and the weight of dry matter was measured.

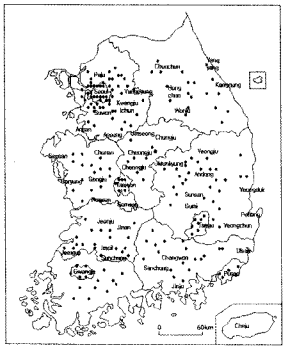
Grouping of γ-producers based on the characteristics of the viscous substance While the production of the viscous substance by isolated strains on the glutamate-dependent γ-PGA production plates was evaluated, the different types of viscous substances were observed. According to the characteristics of the viscous substance, γ-PGA producers were divided into groups.

Photography The plates were photographed using an Olympus digital camera (Olympus, Japan) and the images were processed electronically.

Results and Discussion

Collection of food samples and isolation of microorganisms We isolated γ-PGA producers from 439 varieties of three Korean fermented foods, *Cheongkukjang*s, *Doenjang*s and *Kochujang*s, that were collected from different regions with the aim of isolating various γ-PGA producers with different characteristics. We presumed that the variation in taste and flavor of the fermented foods, which are affected by microorganisms, indicates the presence of different *Bacillus* sp. Therefore, we isolated *Bacillus* sp. from various regional samples across the nation. On the NA plate, four types of colony were formed at most, two of which showed similar morphological characteristics to *Bacillus* sp. We obtained only *Bacillus*-like microorganisms in 217, 272, and 164 isolates from 161 *Cheongkukjang*, 171 *Doenjang*, and 107 *Kochujang* samples, respectively (Fig. 1).

A.



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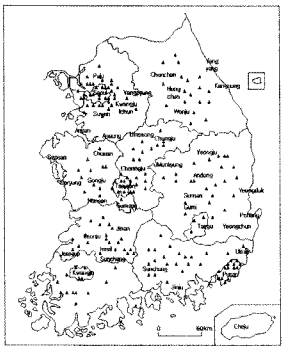

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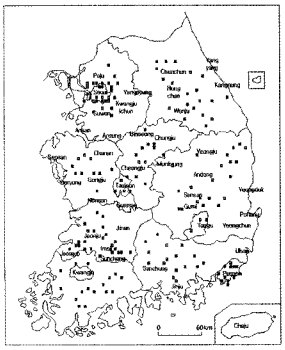


D.

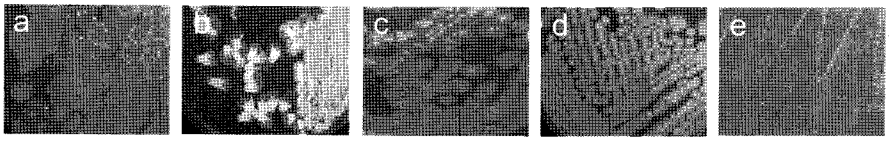
	Cheongkukjang	*Doenjang*	*Kochujang*	Total
Food samples	161	171	107	439
Isolated microorganisms	217	272	164	653

Fig. 1. Collection of food samples and isolation of microorganisms. Food samples of *Cheongkukjang* (A), *Doenjang* (B), and *Kochujang* (C) were collected from different regions. The sampling sites are marked on the map by (●) for *Cheongkukjang*, (▲) for *Doenjang*, and (■) for *Kochujang* samples. The numbers of food samples and isolated microorganisms are summarized (D).

Isolated microorganisms were grouped into five classes based on the level of production of viscous substance in the glutamate-dependent γ-PGA production media The production of γ-PGA is affected by several culture conditions. Some strains require L-glutamate for γ-PGA production while others do not. We evaluated the level of γ-PGA production on the media containing L-glutamate. As shown in Fig. 2, a total of 653 strains were grouped into five classes. The strains of the very high level group produced viscous substances to the extent of overflowing to the lid of the plate (Fig. 2A a). The portion of very high level producers was greater in the strains isolated from *Cheongkukjang* (4.7%) than in those from *Doenjang* (1.8 %) and *Kochujang* (3.0%) (Fig. 2B). The high, medium, and low level producers were found at a similar portion of around 30%, while about 5% did not produce any detectable viscous substance (negative group) (Fig. 2A e).

Isolated microorganisms were grouped into five classes based on the level of production of the viscous substance in the glutamate-independent γ-PGA production media On a plate of glutamate-independent γ-PGA production media, the isolated strains grew slowly and produced less γ-PGA than in the glutamate containing media. About 6% of the strains did not grow and the others were grouped into four classes based on the level of γ-PGA production. The production of viscous substance was not detectable in over 60% of the isolated strains (negative group) (Fig. 3). As shown in Fig 3A, about 6% of strains produced viscous substance that spread from

A.

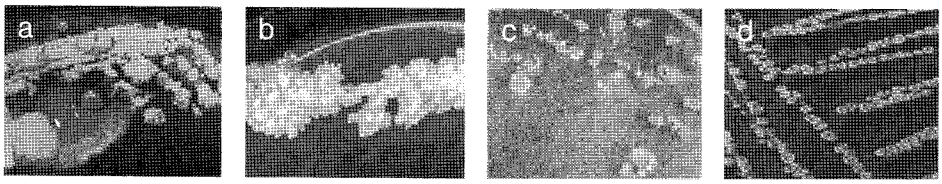


B.

Group	*Cheongkukjang* (217 strains)	*Doenjang* (272 strains)	*Kochujang* (164 strains)	Total (653 strains)
Very high (a)	10(4.7%)	5(1.8%)	5(3.0%)	20(3.1%)
High (b)	71(32.7%)	75(27.6%)	52(31.7%	198(30.3%)
Medium (c)	63(29.0%)	100(36.7%)	44(26.8%)	207(31.7%)
Low (d)	64(29.5%)	75(27.5%)	55(33.5%)	194(29.7%)
Negative (e)	9(4.2%)	17(6.2%)	8(4.9%)	34(5.2%)

Fig. 2. Evaluation of γ-PGA production of isolated microorganisms on the glutamate-dependent γ-PGA production media. The isolated microorganisms were inoculated onto the glutamate-dependent agar plates and grouped into 5 classes (a, very high; b, high; c, medium; d, low; e, negative) based on the qualitative evaluation of γ-PGA production (A). The number of microorganisms belonging to each group is summarized (B).

A.



B.

Group	*Cheongkukjang* (217 strains)	*Doenjang* (272 strains)	*Kochujang* (164 strains)	Total (653 strains)
High (a)	11(5.1%)	16(5.9%)	10(6.1%)	37(5.7%)
Medium (b)	8(3.7%)	16(5.9%)	8(4.9%)	32(4.9%)
Low (c)	35(16.1%)	46(16.9%)	39(23.8%)	120(18.3%)
Negative (d)	149(68.7%)	180(66.2%)	98(59.7%)	427(65.4%)
No growth	14(6.4%)	14(5.1%)	9(5.5%)	37(5.7%)

Fig. 3. Evaluation of γ-PGA production of isolated microorganisms on the glutamate-independent γ-PGA production media. The isolated microorganisms were inoculated onto the glutamate-independent agar plates and grouped into 5 classes (a, high; b, medium; c, low; d, negative; no growth) based on the qualitative evaluation of γ-PGA production (A). The number of microorganisms belonging to each group is summarized (B).

colonies and these strains were designated as high level, while about 5% and 18% of strains were grouped into medium and low level producers, respectively (Fig. 3A b, c and Fig 3B).

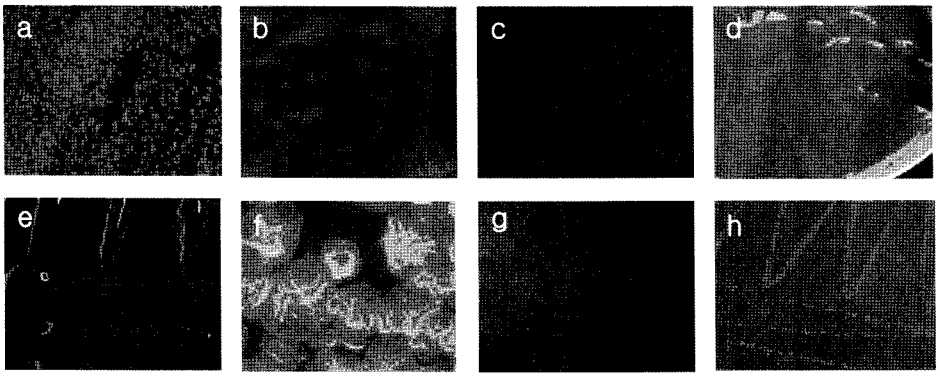
Isolated microorganisms were grouped into eight classes based on the types of viscous substance The isolated strains produced seven different types of viscous substance with no growth being shown in the negative group (Fig. 4). Some strains produced viscous substance in the shape of a grain (Fig 4A a) and others formed along colonies in the shape of a drop (Fig. 4A b). The most frequent type, in one quarter of the strains, was the dispersing viscous substance spread over the surface of the plates (Fig. 4A c). The type of viscous substance shown in Fig. 4A d was similar to that of the dispersive type, but the content was much higher to the extent that it overflowed to the lid of the plates. The transparent type of viscous substance looked like a layer of water (Fig. 4A e). The most unique viscous substance was the jelly-like type that formed a thick, wrinkled layer (Fig. 4A f). The seventh type of viscous substance was the formation of a thin, dried layer over the surface of the plates (Fig. 4A g).

The physicochemical characteristics of the seven different types of viscous substance remain to be elucidated, and several factors such as polymer length and the ratio of D-/L-glutamate may be influential. Some viscous substances may contain other substances in addition to γ-PGA. The hydrolysis of crude γ-PGA yielded mainly glutamate but other substances were observed on TLC (data not shown).

In previous studies, *Cheongkukjang* and other sources have been applied to isolate γ-PGA producers (11), but with a limited number of *Cheongkukjang* samples collected only from a certain region. Because we screened γ-PGA producers from various varieties of *Cheongkukjang*s, *Doenjang*s, and *Kochujang*s collected from different regions, there is a higher probability that the isolated *Bacillus* sp. may produce various types of γ-PGA suitable for certain industrial applications. As expected, different types of viscous substance were observed and their physicochemical properties are expected to vary, which would favor certain industrial applications. In addition, because they were isolated from foods consumed for several millennia, the γ-PGA produced by them can be used for human consumption without an extensive safety evaluation.

The maximum γ-PGA production yields were 32.5 g/L and 5 g/L, depending on the purification methods in the glutamate-dependent media. The higher yield resulted from a simple precipitation of γ-PGA by either methanol or ethanol and dialysis. This crude γ-PGA often contained other substances in addition to γ-PGA, when evaluated by

A.



B.

Group	*Cheongkukjang* (217 strains)	*Doenjang* (272 strains)	*Kochujang* (164 strains)	Total (653 strains)
Grain-like (a)	12(5.6%)	16(5.9%)	14(8.5%)	42(6.4%)
Drop-like (b)	43(19.8%)	36(13.2%)	20(12.2%)	99(15.2%)
Dispersive (c)	51(23.5%)	68(25.0%)	47(28.7%)	166(25.4%)
Overflowing (d)	35(16.1%)	39(14.3%)	16(9.7%)	90(13.8%)
Transparent (e)	20(9.2%)	31(11.4%)	16(9.7%)	67(10.3%)
Jelly-like (f)	17(7.8%)	10(3.7%)	6(3.7%)	33(5.0%)
Thin layer-forming (g)	30(13.8%)	55(20.2%)	37(22.6%)	122(18.7%)
Negative (h)	9(4.2%)	17(6.3%)	8(4.9%)	34(5.2%)

Fig. 4. The types of viscous substance produced by isolated microorganisms on the glutamate-dependent γ-PGA production media. The isolated microorganisms were inoculated onto the glutamate-dependent agar plates and grouped into 8 classes (a, grain-like; b, drop-like; c, dispersive; d, overflowing; e, transparent; f, jelly-like, g, thin layer-forming; h, negative) based on the apparent characteristics of the produced viscous substances (A). The number of microorganisms belonging to each group is summarized (B).

TLC after acid hydrolysis (data not shown). Pure γ-PGA was obtained by further purification employing sulfuric acid and proteinase K treatments to remove carbohydrates and proteins. The γ-PGA production yield of our isolated strains was almost equivalent to that reported by previous studies of 10-35 g/L (14, 18). However, the yield of our isolated strain may be increased after optimization of the γ-PGA production conditions. Therefore, the isolated strains producing various types of viscous substances will serve as a natural resource to screen industrial producers of γ-PGA with desirable characteristics.

Acknowledgments

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