ARTICLE

Trial of Isolating the High Yield Bacteria for Producing Poly-γ-Glutamate With Wheat Bran as Fermentation Raw Material

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＜Abstract＞

Poly-γ-glutamate (PGA) is a major component of the mucilage material of natto together with fructan, and it is a copolymer of DL-PGA. It is a functional material receiving the authorization of a food for specified health use as Ca-sorbefacient from the Ministry of Health, Labour, and Welfare. Since there are various effective characteristics of PGA such as humidity retention characteristics, edible properties and so on, many uses are considered including for cosmetics, the purification of water, as an earth-water preservation agent for desert tree planting, and as raw materials for biodegradable plastic. Now glutamic acid is used as the raw materials to produce PGA by fermentation with ***Bacillus subtilis*** (natto), but we have been working on a way to establish the cheaper production method using wheat bran, which is an industrial by-product. We report in this paper the result of screening bacterial strains which can transform the high protein fraction derived from wheat bran into PGA resulting in a higher yield.

Keywords: poly-γ-glutamate, wheat bran, ***Bacillus subtilis*** (natto)

PGA was found at first in 1937 as a component of capsule for ***Bacillus anthracis***. PGA is allowed legally by the Ministry of Health, Labour, and Welfare of Japanese government to use Ca-sorbefacient in food as the specific health food, since its Ca-binding capacity is demonstrated scientifically[1]. Also it is utilized industrially as a humectant in a solid soap, a toilet water and a cosmetic gel[2]. It is an unusual anionic polyisopeptide in which only glutamate is polymerized via γ-amide linkages. The sticky polymer is the principal component of natto, a traditional Japanese food prepared from steamed soybeans by fermentation with ***Bacillus subtilis*** (natto)[3]. The molecular weight of PGA is maximally 7000kDa[4]. It is a copolymer DL-PGA[5] with a high-molecular-mass L-glutamate-rich fragment (160-400kDa in average) and with a low-molecular-mass fragment composed mostly of D-glutamate residues (5kDa in average)[6].

PGA has various characteristics such as a highly water absorbing ability, a metal-absorbing ability and antifreeze-activity[7,8]. PGA is tasteless, orderless, biodegradable and edible[7]. Recent study suggested that PGA seems to protect baker's yeast from lethal freeze injury, leading to a high leavening

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ability after freezing and thawing[9]. Also, novel application of PGA have been calling for interest in a wide range of industrial use such as medicine, food, cosmetics, a humectant, a water purifier, a crazing inhibitor of the concrete, an earth-water preservation agent for desert tree planting, a dew condensation inhibitor, a solid soap and biomaterials[9-11].

Regarding that PGA becomes an important functional material of many use, the reduction of production method should be carried out according to the progress of its development on effective utilization. Now glutamic acid is used as raw materials to produce PGA by fermentation with *Bacillus subtilis* (natto), but we have been working on to establish the cheaper production method with the wheat bran which is an industrial by-product and is rich in protein possibly can be a source to be PGA by bacterial fermentation. In previous study[12], we found the method to obtain BHPF, the fraction of protein content 21.6% in the yield of 12% from general wheat bran by a combination of the operation of sieving and pin-milling. From BHPF, the amount of the fermentation product equal to that in the case of from gluten was obtained with the use of *Bacillus subtilis* NRRL B-2612 strain for each case. However, the molecular weight of provided PGA was much smaller when compared with PGA got from gluten[13]. To obtain higher molecular weight of PGA from wheat bran, several matters should be examined such as culture condition, composition of culture medium, bacterial strain and so on. Here in this report, the result of our effort on screening the suitable strain from circumstances within Fukuoka prefecture for effective PGA production with wheat bran was shown.

MATERIALS AND METHODS

BHPF

According to the physical isolation method developed by Ranhotora *et al.*[14] was used with little modification. Wheat bran was pin-milled at flow rate of 1 kg/min with Alpin-Itoman160Z (Alpin-Itoman Co., Ltd., Tokyo) at the rotation speed of ML (11200rpm) and shifted with wire sieve (Tokyo Screen Co., Ltd, Tokyo) of 106 micron at the rotation speed of 140rpm and flow speed of 10g/3min. Each sieve through fraction was examined. The sieve through, high protein fraction from wheat bran was named BHPF (Bran High Protein Fraction). Its protein content was 21.6% and its amino acids composition is as shown[12].

Bacterial strain

Bacillus subtilis NRRL B-2612 was used as a control strain in this study. Ward *et al.*[13] succeeded the fermentation production of PGA from wheat gluten using this strain. *Bacillus subtilis* ASAHI was used as a standard strain and it is suitable strain for PGA production from glutamic acid[2].

Medium

SG agar medium[2]: This medium was used for 1st ad 2nd screening. This medium is a glutamate synthetic nutrient medium. The composition was as follows; 25%sucrose, 7.5%Sodium L-glutamate, 0.25%Sodium chloride, 1.35% KH_2PO_4, 2.1%$Na_2HPO_4 \cdot 12H_2O$, 0.25%$MgSO_4 \cdot 7H_2O$, 0.1%Biotin (100 μg/ml), 2.0% agar in well water (tap water is not applicable). Medium was autoclaved at 121℃, 2atm, 15min and plated in the clean bench.

BHPF medium[12]: The composition of pre-culture and the main culture was 1% K_2HPO_4, 0.7%NH_4Cl, 0.0041%$MgCl_2 \cdot 6H_2O$, 0.004% $FeCl_3 \cdot 6H_2O$, 0.015%$CaCl_2 \cdot 2H_2O$, 0.00005%$ZnCl_2$, 0.001% $MnCl_2 \cdot 4H_2O$, pH6.5, and 10% of BHPF. For agar plate, 2.0% agar was added. Medium was autoclaved at 121℃, 2atm, 15min.

Fermentation condition

The liquid culture procedure was followed by Ward *et al.*[13] with a little modification. BHPF was used instead of wheat gluten. The pre-culture was done for 24hrs at 33℃ and 200rpm. 10% of the pre-culture was added to the main-culture. The cultivation was performed for

30hrs at 33℃ and 200rpm.

PGA purification

Purification of PGA from cultured medium was followed by Fujii's method[15] with a little modification. Culture fluid after the culture was centrifuged at 8000rpm for 40min. under 4℃ to separate cells from the supernatant that was collected by decantation into a glass beaker. Ethanol of the twice as much volume as to the supernatant was added little by little into the beaker with mixing slowly with a glass stick, PGA coiled itself around the glass stick. This PGA was air dried until alcohol transpired with having coiled itself around the glass stick and dissolved this PGA in sterilization water again. The PGA solution was dialyzed to cold water with the dialysis tube (UCC cellulose tubing C-110, Shiraimatsu instruments Co. Ltd, Japan) until the OD_{280} of the outer membrane solution became under 0.1. The inner membrane solution was freeze dried and the dried sample was assumed as a purified authentic PGA.

Determination of the molecular weight of purified PGA

The purified sample was dissolved into an acetic acid buffer solution to become 0.1% concentration and HPLC analysis was performed to estimate the average molecular weight of a PGA sample at the experimental condition as follows, Instrument: Shimazu LC10AD, Column: Toso TSKgel G6000PWXL (7.8mmID ×30cm), Eluant: 1M acetic acid buffer solution, Flow rate: 1ml/min, Injection: 10 micro little, temperature 40℃, Detector: RI, Molecular weight standard: polyethylene oxide.

Screening method

Samples for screening a strain that ferments BHPF effectively to produce PGA were collected from rice straw, the soil of the rice field, the soil of the various places, vineyard, persimmon field and peach field within the region and its outskirts, and foreign wheat, barley and rye. A certain farming family in that district was fermenting the homemade natto using a strain which was taken out of rice straw, then the strain of ***Bacillus subtilis*** (natto) was also examined in this study.

1st screening: The sample was placed in the sterilized water for 60 min to introduce its sporulation, then boiled for 20 min to sterilize various germs except Bacilli and other microbial spores. The spore suspension was streaked with a loop on a SG agar plate and incubated for 4 to 5 days at 37℃ . Colonies that produce the mucilage material were selected and used for 2nd screening.

2nd screening: Each strain was cultured on a SG agar plate and the following analytical items were measured; the sticky length of the mucilage material when the colony was pulled with a toothpick, and the wet and dry weight of the mucilage material that was taken out from the surface of the agar plate with a sterilized spurtle. Then the good strains were selected according to the results.

3rd screening: Each strain was cultured in the SG liquid culture, and the yield of PGA and the molecular weight was analyzed. ASAHI strain was used as control, since the strain was best strain to produce PGA in the highest amount and molecular weight that we have so far. Then, each strain was cultured in the BHPF liquid culture, and the yield of PGA was measured.

RESULTS AND DISCUSSION

1st screening

Seventy five samples were examined as shown in table 1. Twenty eight samples were rice straw, ten samples were soils of several rice fields, seven samples were soils of a park and a riverside. sixteen samples were soils of fruits fields (berry, persimmon, and peach). Others were Canadian wheat, rye and barley, American and Australian wheat. fifteen bacterial strains that produced sticky compound around the colony were obtained, and all of them showed the characteristic dry, wrinkled and

milky-white surface, suggesting Bacilli. Though seven strains were obtained from rice straws as expected, much more numbers of strains should be found in rice straws since Japanese traditional natto has been made by naturally occurred ***Bacillus subtilis*** (natto) living in rice straw. It is very strange phenomena that there was a region where no Bacilli was found in the rice straw. Microflora is changing in the Japanese rice fields, probably because of the lots of usage of the pesticides in these recent years. Similar findings were observed for the bread yeasts. Until the twenty years ago, there were so many yeasts were found in fruits such as berry, apple, peach and so on (unpublished data), but our recent study revealed that yeasts disappeared from the Japanese fruits almost entirely (unpublished data). These facts suggest that big change is occurring on microflora in our circumstances.

It was interesting and unexpected that six strains were obtained from Canadian barleys, though no bacteria were screened out from other cereals, including Canadian wheat and rye. The reason is very hard to be presumed. In order to clarify the possible relation to barley's constituents, Japanese barley and its field soil should be examined for further study.

2nd screening

Analytical results of selected fifteen strains were shown on table 2. There are marvelous differences in the characteristics observed among the screened strains. Thirteen strains showed longer sticky length than the control strain ASAHI. Eleven strains exhibited the bigger weight than the control strain. There was weak correlation between sticky length and weight of mucilage materials. It is suggested that longer sticky length means more involvement of both PGA and fructan since they are the materials that cause the stickiness, indicating the higher purity of those materials. Therefore, taking both features into account, eight strains were selected for further analysis, and they were named as NEBA1 to NEBA8 as shown in table 2.

Table1. The result of 1st screening

Sampling area and number		Strains	Sampling area and number		Strains
Rice straw around	①	2	Soil of a park in	①	0
Yoshii town	②	2	Kurume city	②	0
	③	0		③	0
	④	0		④	0
	⑤	0	Soil of riverside around	①	1
	⑥	1	Haraduru onsen	②	0
	⑦	1		③	0
	⑧	0	Soil of berry field around	①	0
	⑨	0	Yoshii town	②	0
	⑩	0		③	0
	⑪	0		④	0
	⑫	0	Soil of percimmon field	①	0
	⑬	1	around Yoshii town	②	0
	⑭	0		③	0
	⑮	0		④	0
	⑯	0		⑤	0
	⑰	0		⑥	0
	⑱	0	Soil of peach field	①	0
	⑲	0	around Ukiha town	②	0
	⑳	0		③	0
Rice straw around	①	0		④	0
Tanushimaru town	②	0		⑤	0
	③	0		⑥	0
	④	0	Canadian 1CW wheat	①	0
	⑤	0		②	0
	⑥	0		③	0
	⑦	0		④	0
	⑧	0	Canadian barley	①	3
Soil of rice field around	①	0		②	1
Tosu city	②	0		③	2
	③	0	American WW wheat	①	0
	④	0		②	0
Soil of rice field around	①	0		③	0
Hita city	②	0	Australian ASW wheat	①	0
	③	0		②	0
	④	0	Canadian rye	①	0
	⑤	0		②	0
	⑥	0			

1) Numerous number of "strain" undicates the obtained individual colony.
2) Ten agar plates were used for each individual sample.

Table2. The result of 2nd screening

Strain	Sticky length (%)	Wet weight (g)	Dry weight (g)	Naming
ASAHI	100	2.34	0.30	
1	130	1.91	0.22	
2	185	8.33	0.53	NEBA-2
3	175	8.45	0.43	NEBA-3
4	150	5.73	0.41	
5	180	6.29	0.51	
6	3	0.00	0.00	
7	190	6.68	0.33	NEBA-4
8	43	0.75	0.03	
9	140	5.96	0.57	
10	183	6.53	0.52	
11	167	6.18	0.56	NEBA-5
12	200	5.51	0.48	NEBA-7
13	133	8.66	0.61	
14	192	8.88	0.70	NEBA-1
15	180	3.01	0.22	NEBA-8

1) Sticky length was expressed by % compared with that of ASAHI strain as 100%.
2) Wet and dry weight are the average of two agar plates (φ9.0cm).
3) Numerous number of "Strain" is aquired in order of from a beginning of Table1.
4) Naming number of NEBA is aquired with a good turn of the average between "Sticky length" and "Dry weight".

3rd screening

Liquid cultivation was performed for each selected eight strains using L-glutamate as a raw material for PGA production, and the accurate PGA yield and the molecular weight was compared with the features of the control strain ASAHI. The results were shown on table 3. There was no strain that produced more PGA than the control strain ASAHI. Molecular weight of PGA produced by each strain was almost similar among the eight selected strains and the control strain. Table 4 shows the result of PGA yield using BHPF as a raw material for PGA production. Unfortunately, there was no strain that produced PGA more than NRRL B2612 strain.

Table 3. Characterisrics of PGA produced with SG liquid medium

Strain	PGA Production (g dry weight/100ml medium)	Molecular Weight ($\times 10^4$)
ASAHI	0.88	315
NEBA-1	0.57	329
NEBA-2	0.82	333
NEBA-3	0.39	347
NEBA-4	0.68	313
NEBA-5	0.68	320
NEBA-6	0.67	359
NEBA-7	0.51	318
NEBA-8	0.31	289

1) "PGA Production" and "Molecular Weight" are the average of two experiments.

Table 4. PGA production with BHPF liquid medium

Strain	PGA Production (g dry weight/100ml medium)
ASAHI	0.00
NEBA-1	0.56
NEBA-2	0.00
NEBA-3	0.00
NEBA-4	0.00
NEBA-5	0.00
NEBA-6	0.00
NEBA-7	0.10
NEBA-8	0.20

1) Data is the average of two experiments.

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原　著

小麦ふすまを発酵原料にした ポリ-γ-グルタミン酸高率生産菌単離の試み

甲斐　達男

＜要　旨＞

ポリ−γ−グルタミン酸（PGA）は、フラクタンとともに納豆粘質物の主成分であり、DL−PGAのコポリマーである。Ca吸収促進剤として厚生労働省より特定保健用食品の認可を受けている機能性物質である。保湿性が高く可食性であるなどのさまざまな有効な特性があるため、化粧品、水の浄化剤、砂漠緑化のための保水剤、生分解性プラスチックの原料など、多くの用途が検討されている。現在は、グルタミン酸を原料にして、***Bacillus subtilis*** (natto) による発酵生産が行われているが、より安価な生産を目指して、われわれは、産業副産物である小麦ふすまを原料にした生産法を検討してきた。本稿では、小麦ふすまから抽出した高タンパク成分（BHPF）を、高率でPGAに資化できる菌のスクリーニングを行った結果を報告した。

キーワード：ポリ−γ−グルタミン酸、小麦ふすま、*Bacillus subtilis* (natto)

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