原　著

Study on Fermentation Production of Poly-γ-Glutamate with the Industrial By-product Wheat Bran

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<Abstract>

Wheat bran is an industrial by-product in the flour milling industry, and it is used mainly as feed. Wheat bran accounts for about 23% (1,300,000t a year) of the industrial by-products of the wheat during the milling of flour. This is equivalent to about ¥40,000,000,000 annually. Because such industries as bread / noodles / confectionery utilize wheat flour in Japan that the price of the wheat flour has been controlled by the food control system of the country where it was formed, the flour milling industry survives with a low profit margin. When cheap wheat bran flows in by the sudden change of the exchange rate from foreign countries (mainly, the United States), there is the actual situation that the sales decrease amount of money of the minute when the price of the wheat bran fell just suppresses the profit of the company. Actually, it has caused the bankruptcy of many small businesses whenever the yen appreciates. The Japanese Government promotes the collection to the gathering together of the best people in such companies to plan the reinforcement of the corporate culture of the flour milling industry, but it is unchanged that the price fluctuation of wheat bran has a serious influence on the stability of the flour milling industry. Therefore, it could contribute to Japanese flour milling and the allied industrial stabilization if an added value besides a feed use could be given to wheat bran. This report focuses attention on wheat bran, including its richness of amino acids, and the possibility of wheat bran was examined whether it can be a fermentation production raw material of poly-γ-glutamate (PGA) which receives the authorization of Ministry of Health, Labour and Welfare as a health material for the Ca absorption promotion. The high protein fraction (BHPF) of protein content of 21.6% from general wheat bran was able to obtained in a yield of 12% by a combination of the operation of sieving and pin-milling, in this study. The protein content of the high protein fraction was comparatively low by the high-speed pin-milling, but a moderate shock of the degree that did not crush the pericarp in itself was guessed by wheat bran to separate the aleurone layer efficiently when it was necessary. With this BHPF as a raw material, PGA was provided by fermentation production with *Bacillus subtilis* NRRL B-2612 strain. However, impurities (supposed to be glucan) of 18% were detected, and it was not amino acid composition of glutamic acid 100% more. The molecular weight was about 200,000 which was considerably a small molecule in comparison with provided PGA with more than 3,000,000 produced from pure glutamic acid as a fermentation material. The most suitable method to get a fraction of wheat bran of the high protein, and the best culture condition to get high quality PGA are expected to be established in future.

Key words: wheat bran, industrial by-product, poly-γ-glutamate

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Wheat bran is an industrial by-product in the flour milling industry, and it has been used mainly as feed. As for the wheat as the flour milling raw materials, about 5,500,000t a year has been consumed for these past 10 years, and the about 23% that is about 1,300,000t wheat bran has been produced every year and this will form about 40,000,000,000 yen markets[1]. Because a price of the wheat is managed in the flour milling industry by the government, and total industry to surround the wheat flour in our country that the price of the wheat flour has been controlled with it was formed, resulting in such business condition of small profits and quick returns, and there is the actual situation that the sales decrease amount of money of the share that the price of the wheat bran fell just presses the profit of the company directly. When cheap wheat bran flows in by the change of a sudden exchange rate from the foreign countries (mainly, the United States), domestic flour milling industry will suffer a big blow. Actually, it caused the bankruptcy of many small businesses whenever appreciation of the yen happened till now. The Japanese Government promotes the collection to the gathering the best people company not the relief of the small business to plan the reinforcement of the corporate culture of the flour milling industry. The survival of the medium and small-sized business is the extremely difficult situation.

The price fluctuation of the wheat bran will still have a serious influence on the stability of the flour milling industry in future Japan because the large rise of the wheat flour price can not be expected, even if, furthermore, the collection of the company advanced if we think about bread made, noodles made, the stability of the confectionery industry. Therefore, it is possible to contribute to stabilization of the Japanese flour milling industry, if an added value besides a feed use could be given to wheat bran. Therefore in this report, paying the attention to a wheat bran including an amino acid in richness, the possibility of wheat bran as fermentation production raw materials of poly-γ-glutamate (PGA) are studied

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[2]. Also it is utilized industrially as a humectant in a solid soap, a toilet water and a cosmetic gel[3]. It is an unusual anionic polyisopeptide in which only glutamate is polymerized viaγ-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)[4]. The molecular weight of PGS is maximally 7000kDa[5]. It is a copolymer DL-PGA[6] 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)[7].

PGA has various characteristics such as a highly water absorbing ability, a metal-absorbing ability and antifreeze-activity[8,9]. PGA is tasteless, orderless, biodegradable and edible[8]. Recent study suggested that PGA seems to protect baker's yeast from lethal freeze injury, leading to a high leavening ability after freezing and thawing[10]. 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[10-12].

MATERIALS AND METHODS

Analysis of crude protein content

Kjeltec2300 automatic analyzer (FOSS· JAPAN CO., Ltd, Tokyo) was used. The analytical principle is based on Kjedahl method.

Amino acid quantification

HPLC (LaChrom Elite Personal HPLC System, Hitach High-Technologies Corporation, Tokyo) was used with the method of the reversed-phased chromatography for amino acid quantification. Experimental samples were pre-treated with HCl hydrolysis method and pre-labeld. 20mg sample was hydrolyzed with 2ml of 6N HCl for 20hrs at 110˚C

and phenyl-isothiocyanate (PITC) was labeled by conventional method. For HPLC column, PICO-TAG™ was used. PTC-Amino Acids Mobile Phase A and Phase B (Wako Co., Ltd., Tokyo) were used as eluants at linear gradient of 0 to 70% of Phase B for 15min. at 40˚C. 10 micro little sample was injected and UV detector was performed at 254 nm (0.32AUFS).

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 G6000 PWXL (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.

Quantification of reducing sugar

Lane-Eynon Method[13] was performed. The method is applicable to all starch hydrolysis products. At the Lane and Eynon constant titre method, mixed Fehling's solution is titrated with sample using methylene blue as indicator. This method is the International Standard: ISO5377.

Wheat bran

General wheat bran is commercially circulated in the market and its material wheat species are mixed. Therefore the nutritional composition is different by lots. In this study, single lot's product was used to get rid of an error to get together between lots. Also, wheat bran which is isolated from the individual wheat brand were used, that is, 1CW (No.1 Canada Western Red Spring Wheat), ASW (Australian Standard White), WW (White Wheat) and DW (Domestic Wheat) that can not be obtained in the market, but in this study, those were supplied from a commercial company for their kindness. These wheat brands are the most generally used in Japanese milling industry.

Extraction of high protein fraction from wheat bran

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) and shifted with wire sieve (Tokyo Screen Co., Ltd, Tokyo). The rotation speed of pin-mill was tested for 4 different speeds of L (9000rpm), ML (11200rpm), MH (14000rpm) and H (18000rpm). The inner diameter of the sieve was 19.7mm and the multi-stage sieve with the mesh of 1180, 710, 600, 425, 300, 212 and 106 micron series were used at rotation speed of 140rpm and flow speed of 10g/3min. Each sieve through fraction was examined. The extracted high protein fraction from wheat bran was named BHPF (Bran High Protein Fraction).

Bacterial strain

Bacillus subtilis NRRL-B-2612 was used. Ward *et al.*[15] succeeded the fermentation production of PGA from wheat gluten using this strain.

Fermentation condition

The liquid culture procedure was followed by Ward *et al.*[15] with a little modification. Wheat bran and BHPF were used instead of wheat gluten. The pre-culture was done in 2% gluten and 0.5% K_2HPO_4 medium for 24hrs at 33˚C and 200rpm. 10% of the pre-culture was added to the main-culture. Composition of 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 5-15% of wheat bran or BHPF was added. Both the pre-culture and the main-culture were autoclaved at 121˚C, 2atm, 15min. Cultivation was performed for 30hrs at 33˚C and 200rpm.

The medium composition was same as the liquid culture for standstill cultivation. 50g, 100g or 200g of gluten, wheat bran or BHPF was placed in the 1 litter beaker and 50ml of main-liquid culture (prepared as same as the liquid cultivation) was added and autoclaved. Then, 5ml of pre-culture (prepared as same as the liquid cultivation) was added and cultured at 33˚C for 24hrs, 48hrs and 72hrs, individually.

Right after cultivation, 200ml of sterilized distilled water was added to the medium, mixed vigorously for 1 hr for extraction of PGA.

PGA purification

Purification of PGA from cultured medium was followed by Fujii's method[16] with a little modification. Culture fluid after the culture was centrifuged at 8000rpm for 40min. under 4˚C 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.

RESULTS AND DISCUSSION

Separation of high protein fraction (BHPF) from wheat bran

Really various studies have been made about the method to separate wheat protein till now by a wheat conponent. The industrialized method for gluten preparation from wheat is so called the wet-separation method. The method is based on utilizing such the gluten property that gluten protein with special viscoelasticity can be separated from the dough to starch easily by washing in water[17]. In addition, following methods were developed; (1) protein elution method to liquefy protein by alkali, (2) separate the starch or protein with centrifugation by liquefying starch or protein with an enzyme[18], (3) the method utilizing the difference in a specific gravity between protein and starch a specific gravity, with the use of a mixture solvent of benzene and carbon tetrachloride.

The air-classification method is studied best for a physical separation method. The principle is the things to cut both in air-classify by a difference of the shape to be a round ball type as for the starch whereas a difference and the protein of specific gravity (1.3) of the protein and the specific gravity (1.5) of the starch are wedge types. There is a problem effectively, but is tried as for the electrostatic separation method that used protein and a difference of the electrostatic charge of the starch grain[19].

Because the formation of gluten does not do it, in the case of wheat bran, the gluten cannot be obtained from wheat bran by the application of gluten separation method. According to the alkali method and the enzyme method, the effective separation of the bran protein seems to be possibility, but include the drainage processing, and the adoption of the wet method is not realistic because considerable facilities cost is necessary. In addition, thinking about efficiency of the production because it was desirable to work it into the existing process of the mill hand hall, the physical separation method was examined in this study.

Wheat bran consist of pericarp /aleurone layer/ germ. The pericarp and aleurone layer hold most judging from the constitution ratio from the part of the endosperm, and there is approximately equal amount as for both. Aleurone layer are high protein, and, as for the protein content of pericarp[20], the separation of high protein fraction will aim at the separation of aleurone layer (18 or more 32.3% protein) from pericarp (around 4% protein). This is supposed when separation of both is formed by giving wheat bran a shock, and separation by air-classification is already tried[14], and it is separated from Hard Red Winter Wheat by efficiency of edible produce 10% fraction of protein 20%[15].

Wheat bran was pin-milled at four different rotation speed of L (9000rpm) / ML (12000rpm) / MH (14000rpm) / H (18000rpm). This result was shown in Table 1, but, as for every bran tested, the high protein fraction of protein content around 20% was provided in the through of the sieve of 106 micron. Other all fractions showed less than 12% protein content,

demonstrating that 106 micron through fraction is the most suitable material for PGA production by fermentation. This accords with high protein fraction having been provided on the small part of the grain by the experiment of Ranhotra[13]. As rotation speed increased, yield of this fraction increased with 9.8, 12.0, 16.3 and 25.8%. However, protein content changed with 20.8, 21.6, 19.2 and 18.4% and the bran which was handled in ML exhibited the fraction of the most high protein content (Table 1). The degree of the increase of protein content did not always accord with increase of yield, but the tendency that was similar on the test according to the different wheat raw materials was shown in this way so that it was shown in Figure 1.

The method that is the most effective to get high protein fraction is the thing which gathered through which a decoration is in through of sieve of 106 micron after a pin-milling in ML (12000rpm) and divided (BHPF). About BHPF of general wheat bran, the result that performed amino acid analysis is shown to Figure 2. This result shows that contents of glutamic acid and the alanine which can become the fermentation production raw materials of PGA increase significantly.

Table 1. The yield and the protein content of each fraction that were sieved with step-wised meshes after pin-milled at four different rotation speed.

	1180μ	710μ	600μ	425μ	300μ	212μ	106μ
L (9,000rpm)	0 (-)	16.1 (6.4)	6.2 (6.7)	57.4 (6.6)	8.3 (7.8)	2.2 (10.6)	9.8 (20.8)
ML (11,200rpm)	0 (-)	5.8 (7.5)	5.4 (7.8)	55.9 (7.9)	9.1 (8.3)	11.8 (11.4)	12.0 (21.6)
MH (14,000rpm)	0 (-)	5.2 (6.1)	8.1 (6.5)	42.8 (6.8)	7.1 (7.6)	20.5 (9.0)	16.3 (19.2)
H (18,000rpm)	0 (-)	2.9 (5.3)	6.2 (5.8)	32.5 (6.3)	10.4 (7.4)	22.2 (8.2)	25.8 (18.4)

*Numerous values without parenthesis is yield (%) and those in parenthesis are protein content of each fraction shown on the basis of 14% moisture level.

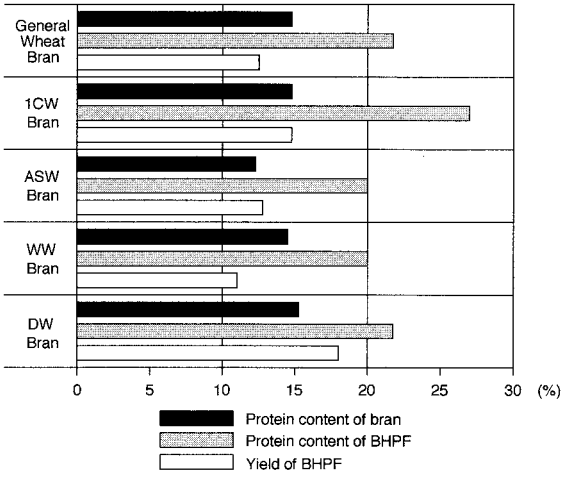

Figure 1. The protein content of various bran and the BHPF with their yield.

Amino acid analysis of BHPF and wheat bran

At first, the value of the raw materials to make good use of an industrial by-product of wheat bran as fermentation production raw materials of PGA has to be examined. According to Fujii[21], the amino acid becoming the raw materials of the PGA production by *Bacillus natto* is done with L-glutamic acid / L-aspartic acid / DL-alanine, and production of the some mucilage thing by *Bacillus natto* is recognized from L-asparagine and L-proline. Therefore, protein content of use raw materials and the content of these amino acids were analyzed on these, because those were thought with a good index to know the raw materials value.

As for general wheat bran, width comes out to the chemical composition by a difference of lot which did sampling so that it is drained with the form that bran was mixed from various raw materials wheat from a flour milling factory. Amino acid analysis of the major wheat bran produced in Japan is shown in Table 2. It was shown that the BHPF

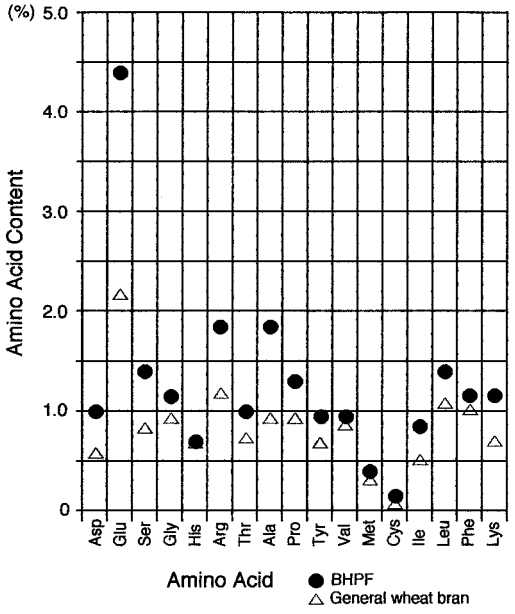

Figure 2. Amino acid content of BHPF derived from general wheat bran.

of the WW origin is useful as PGA fermentation raw materials, since the contents of glutamic acid / aspartic acid when compared among four kinds of bran were considerably high. Content according to raw materials had little blurring about alanine, but considerable blurring was thought to be glutamic acid about aspartic acid. This will reflect quality blurring of general wheat bran as PGA fermentation raw materials.

The examination of the fermentation production condition of PGA

About the liquid culture, as for the experiment of control gluten[15], an approximately equal yield were provided so that it was shown in Figure 3. From BHPF, PGA of the yield of two or three times was provided for bran of no processing. As for the yield per nutrient medium 1ml, a yield equal to the 5.6g neighbor, 10% gluten nutrient medium was provided in the 15% BHPF nutrient medium. Furthermore, the greatest yield was provided at the time of culture by the standstill culture method for 48 hours so that it was shown in Table 3. The amount of water added to the protein raw materials in the nutrient medium was equal in no processing bran, and the PGA yield at the maximum was provided in two times volume of water for gluten and BHPF. As for the production efficiency per the raw materials gram, a value equal to the liquid culture method in the composition nutrient medium using pure glutamic acid was provided in gluten.

The crude protein content of the mucilage substance produced by fermentation was 82%, and remaining 18% were estimated by analysis of Lane-Eynon Method when it was sugar. As for the amino acid composition of the crude protein, glutamic acid held 67%, but the remainder was occupied in other amino acids. It was a small molecule about 200,000 as for the molecular weight, quite different from 3,500,000 derived PGA from wheat gluten that was

Table 2. The contents of crude protein for major wheat bran produced in Japan ad their amino acid contents that can be utilized for PGA production by fermentation.

	Crude Protein	Glu	Asp	Ala	Total
General Wheat Bran (13.2)	14.2	2.2	0.6	0.9	3.7
1CW Bran (13.3)	14.8	2.1	0.6	0.9	3.6
ASW Bran (13.3)	12.3	1.9	0.6	0.7	3.2
WW Bran (14.1)	14.3	2.9	1.2	0.8	4.9
DW Bran (12.7)	15.4	2.7	0.6	0.9	4.2

*Numerous values were shown in % on the basis of 14% moisture level, except those in the parenthesis that were shown on as is %. Total means the sum of three amino acids indicated in the table.

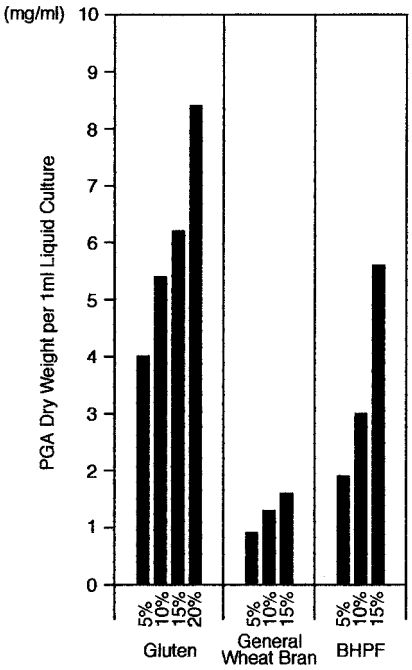

Figure 3. The content of PGA produced by liquid fermentation process for three different raw materials when quantity of addition was varied.

Table 3. The content of PGA produced by stand-still fermentation process for three different raw materials when quantity of addition and the fermentation time were varied.

	Gluten			General Wheat Bran			BHPF		
	25g	50g	100g	25g	50g	100g	25g	50g	100g
24hrs	28.0	31.9	62.3	0.1	0.6	0.4	11.8	26.0	76.2
48hrs	32.4	46.0	84.2	0.3	14.8	14.2	27.1	36.4	76.3
72hrs	29.1	42.9	72.2	0.2	12.8	10.5	16.3	32.7	70.0

*Numerous numbers are PGA dry weight (mg) produced from 1g of raw material. Raw materials examined are gluten, general wheat bran and BHPF with the use of 25g, 50g, or 100g to 50ml liquid medium. Culture time was varied for 24hrs, 48hrs and 72 hrs.

reported by Ward *et al.*[15].

CONCLUSION

Ranhotra *et al.*[14] obtained the fraction of protein around 20% in edible produce of 10% from wheat bran by air-classification method. In this study, by a combination of the operation of sieving and pin-milling at the rotation speed of ML, it was able to get a high protein fraction of protein content 21.6% in the yield of 12% from general wheat bran. Though protein content of the high protein fraction obtained from the high-speed pin-milling was comparatively low, it was guessed that the shock that the degree that did not crush pericarp in itself was moderate was necessary by wheat bran to separate aleurone layer efficiently. From high protein fraction provided from general wheat bran, a fermentation product equal to gluten by *Bacillus subtilis* NRRL B-2612 strain was provided. However, the impurity of 18% glucan was detected by provided fermentation mucilage substance. In addition, the amino acid composition was 67% in the crude protein.The molecular weight of provided PGA was much smaller when compared with PGA which got from gluten[15]. The most suitable method to get fraction of wheat bran of the high protein, and the best culture condition to get high quality PGA are expected to be established in future.

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産業副産物である小麦ふすまからのポリ-γ-グルタミン酸の発酵生産に関する研究

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＜要　旨＞

小麦ふすまは製粉産業における産業副産物であり、主に飼料として利用されている。製粉原料としての小麦の約23%である130万トンが、産業副産物である小麦ふすまとして発生する。これは金額にしておよそ400億円に相当する。国の食糧管理制度によって、小麦粉の価格が抑制された状態で小麦粉を取り巻く製パン・製麺・製菓などの産業が成立してきたために、製粉産業は利益幅の薄い業態をとっている。急激な為替相場の変動によって安価な小麦ふすまが海外（主に合衆国）から流入すると、小麦ふすまの価格が低下した分の売り上げ減少金額がそのまま直接に企業の利益を圧迫するという実態がある。実際、これまで円高が起こる度に、多くの零細企業の倒産を招いてきた。日本政府は製粉業界の企業体質の強化を図るために少数精鋭企業への集約化を推進してきているが、小麦ふすまの価格変動が製粉業界の安定性に甚大な影響を与えてしまうことに変わりはない。従って、小麦ふすまに、飼料用途以外に付加価値を与えることができれば、日本の製粉およびその関連産業の安定化に寄与することができる。本稿では、小麦ふすまがアミノ酸を豊富に含むことに着目して、Ca吸収促進の健康素材として厚生労働省の認可を受けているPGAの、発酵生産原料としての可能性を検討したので、その研究結果を報告した。今回、ピンミル粉砕と篩い分け操作の組み合わせによって、一般ふすまから12%の歩留りで、蛋白含量21.6%もの高タンパク質画分 (BHPF)を得ることが出来た。高速粉砕では高蛋白質画分の蛋白合量は比較的低かったが、小麦ふすまからアリュウロン層を効率良く分離するには、果皮自体を粉砕しない程度のほど良い衝撃が必要であると推察された。このBHPFを原料にして、*Bacillus subtilis* NRRL B-2612 株による発酵生産によってPGAが得られた。しかしながら、18%もの不純物（グルカンと推測される）が検出され、さらに、アミノ酸組成グルタミン酸100%ではなかった。分子量についても約20万と小さく、グルタミン酸を原料して得られる300万を超えるPGAに比べかなり小分子であった。今後、小麦ふすまから高蛋白質の画分を得る方法と、良質のPGAを得るための最適培養条件および精製方法の確立が期待される。

キーワード：小麦ふすま、産業副産物、ポリ－γ－グルタミン酸

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