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Specific gene expression analysis in *Aspergillus oryzae* grown in wheat bran without starch.

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A starch removal method from a wheat bran medium was established using citric acid. Secreted enzymes from cultivated *A. oryzae* RIB40 were then investigated. The specific activity of α-amylase decreased while that of xylanase and xylosidase increased 2-fold and 7-fold, respectively. Proteomics analysis identified three new α-glucosidases and two new α-glucanases. DNA microarray identified high expression of three new endo-glucanases and one α-xylosidase. The expression of β-1,4-xylanase was increased 27-fold.

Keywords:*Aspergillus oryzae*; Wheat bran; DNA microarray; Proteome

Introduction

Wheat is one of the three major cereals in the world. Wheat bran is a by-product of flour milling and over 500,000 tons per year are discharged as bran. Although some wheat bran is used as health food,[1,2] most serves as feed or food waste.[3,4] Considering the global food problems, it is important that food wastage is reduced and an effective use for excess bran is designed. Wheat bran

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is a cell-wall constituent of wheat, containing hemicellulose, cellulose, starch and lignin.[5-7]Approximately 8% of wheat brans are starches. We report on an *A. oryzae* strain, cultivated on solid media, after starch removal from wheat bran.

Aspergillus oryzae is a safe filamentous fungus[8,9], utilized in traditional Japanese fermentation food industries.[10] *A. oryzae* produces and secretes various enzymes into its growth medium.[11-13] Analysis of the genome sequence of *A. oryzae* was completed in 2005.[14] DNA microarray and proteomics analysis were used to detect genes involved in starch breakdown in *A. oryzae* when grown in a wheat bran medium.[15-17] Previously, *Aspergillus* strains containing expression vectors have been used, including a high-producing xylanase strain.[18,19] We report on *A. oryzae* RIB40 using cultivation conditions which did not induce amylase as we aimed to isolate new hemicellulase and cellulose genes.

Materials and Methods

Strains, media and culture conditions

Aspergillus oryzae RIB40 was used throughout this study. *A. oryzae* RIB40 was isolated in 1950 from cereal (raw material of *shoyu*) in Kyoto. All the cultivations were inoculated with 3.3×10^9 conidia of *A. oryzae* RIB40, and incubated at 30 °C for 72h. Wheat bran solid culture was cultivated using 2 g of dry wheat bran with 1.2 ml of water.

Preparation of wheat bran medium without starch

During initial cultivation using starch-containing medium, amylase-like enzymes, such as α-amylase and glucoamylase, were induced and hydrolytic enzyme expression was weak, making proteomics analysis difficult. Therefore, starch removal from wheat bran was performed. First, 2 g of wheat bran was added to 20 ml of 50 mM citrate or water before autoclaving at 121 °C for 20 min. The media was called “A”. Then, 100 ml boiling water was added, before the media was filtered using a wire mesh filter (3 mm x 1 mm) Residual substances were immersed in

100 ml of water for 1 minute. The media was called “AC”.

After 1 minute, the AC media was washed with 300 ml of water and residual substances were collected. These residual substances were washed 5 times with 40 ml of water. This “AC” wash media was called “AC+wash” (Fig.1). Furthermore, culture medium using water instead of citric acid solution was also prepared. Untreated wheat bran medium was used as a control.

Sample collection method

Samples were collected from solid culture using extraction buffer (40 ml of 10 mM acetic acid buffer (pH 5.0), 1 g of 0.5% NaCl, and 160 ml sterile water). Filtration was performed using 90 mm filter paper (Advantec).

Protein determination

The protein concentration in each sample was measured using the Bradford assay and bovine serum albumin as the standard (Bio-Rad).

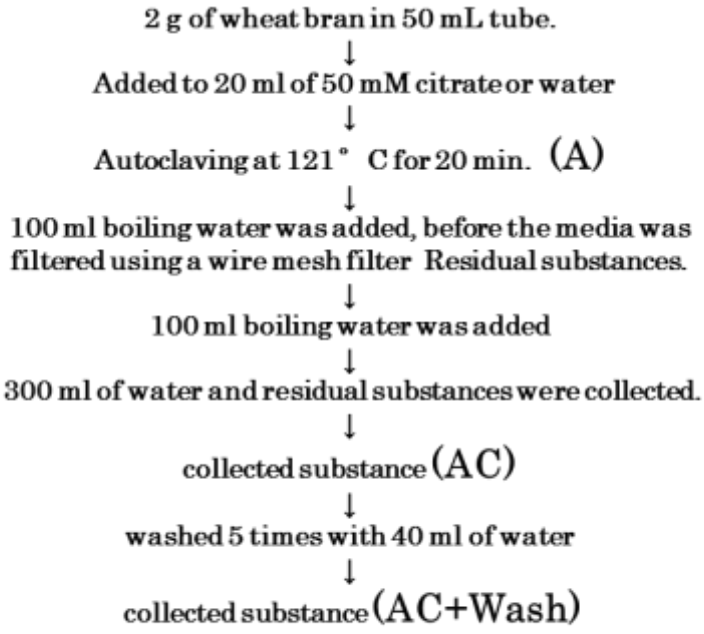

Fig. 1. Preparation of wheat bran medium.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli.[20] Enzyme samples were denatured in 5% (w/v) SDS by incubation for 5 min at 96 °C.

Enzyme activity assays

α-Amylase activity was determined using an α-amylase assay kit (Kikkoman Corp.).[21] In order to perform diastatic power determination, the diastatic-power-assay kit (Kikkoman) was used. One unit of α-amylase activity was defined as the amount of enzyme required to release 1 μmol of 2-chloro 4-nitrophenyl (CNP) from 2-chloro 4-nitrophenyl 6-azide-6-deoxy-β-maltopentaside (N3-G5-β-CNP) per minute at 37 °C.

Xylanase activity was measured using Xylanzyme AX tablets (Megazyme). Detection of β-xylosidase activity used the substrate *para*-nitrophenol-β-D- xylopyranoside (Sigma) and the method of Ait *et al*.[22]

HPLC analysis conditions

Quantitative analysis of xylose, obtained using two-step enzymatic treatment, and arabinose was performed using HPLC under the following conditions[23]: alliance dissolution test system 2695 (Waters), RI detector 2414 (Waters) and a Shodex SPO 810 column. The mobile phase was water, flow velocity of 400 μL/min, temperature 65 °C, and analysis time of 35 minutes. The retention time of xylose was 24-26 minutes, and arabinose was 28-30 minutes.

Two-dimensional electrophoresis

Protein concentration was measured using the Bradford method. Samples were adjusted to give 300 μg of protein for two-dimensional electrophoresis analysis, performed as reported previously. [23]

mRNA preparation

Total RNA was isolated from liquid-grown mycelia using ISOGEN(Wako, Osaka, Japan), according to the manufacturer's instructions. Briefly, mycelia were

ground in liquid nitrogen to a fine powder, and 1 g was mixed with 15 ml ISOGEN solution. After addition of 3 ml chloroform, samples were vortexed then centrifuged at 5000 g for 20 min. The supernatants were transferred to 7.5 ml isopropanol in a fresh tube and the mixture was left at room temperature for 10 min before centrifugation at 5000 g for 20 min. The pellet was rinsed in 100% ethanol, and centrifuged again at 5000 g for 5 min. The pellet was dissolved in 1 ml of RNase-free water and stored at -80 °C until use.

Total RNA was isolated from mycelia cultured on solid medium according to the modified methods of Cathala *et al*.[24].

DNA microarray experiments

DNA microarray experiments were performed as described previously.[25] Two cDNA samples labeled with either Cy5 and Cy3 were mixed and applied onto 12 K *A. oryzae* oligonucleotide microarrays (Fermlab/NovusGene, Tokyo, Japan) for hybridization. The slide was scanned to measure the fluorescence intensity of the two fluorophores using a GenePix 4200A scanner (Axon Instruments at Molecular Devices, Sunnyvale, CA). Analysis was conducted using DOGAN at the National Institute of Technology and Evaluation (NITE). (http://www.bio.nite.go.jp/dogan/project/view/AO).

Results

Starch removal from wheat bran by solubilization

A. oryzae RIB40 was incubated in A, AC and AC+Wash media, with or without citric acid, to remove starch. The starch removal was performed using A, AC and AC+wash. When starch removal was performed using water, a 50kDa protein, assumed to be α-amylase, was detected. In comparison with the control, a small decrease in the 50 kDa α-amylase band was observed when A and AC were used. A greater amount of α-amylase was removed when AC+wash was used. Much larger decreases were observed when citric acid solution was used in the washes.

Acidic hydrolysis of wheat bran using 50mM citrate, autoclaving (121 °C, 20 minutes) and hot water washes removed most of the starch, probably due to decomposition removal (Fig. 2). Accordingly, α-amylase was not confirmed in the around 50kDa as a result of proteome analysis (data not shown). In addition, the effectiveness of starch removal was also confirmed by measuring specific enzyme activities (Table 1).

The specific activity of α-amylase decreased and the activities of xylanase and xylosidase increased in *A. oryzae* RIB40 when cultivated in AC and AC+Wash with citric acid. AC+wash with citric acid decreased α-amylase activity and diastatic power when compared with the control (Table 1).

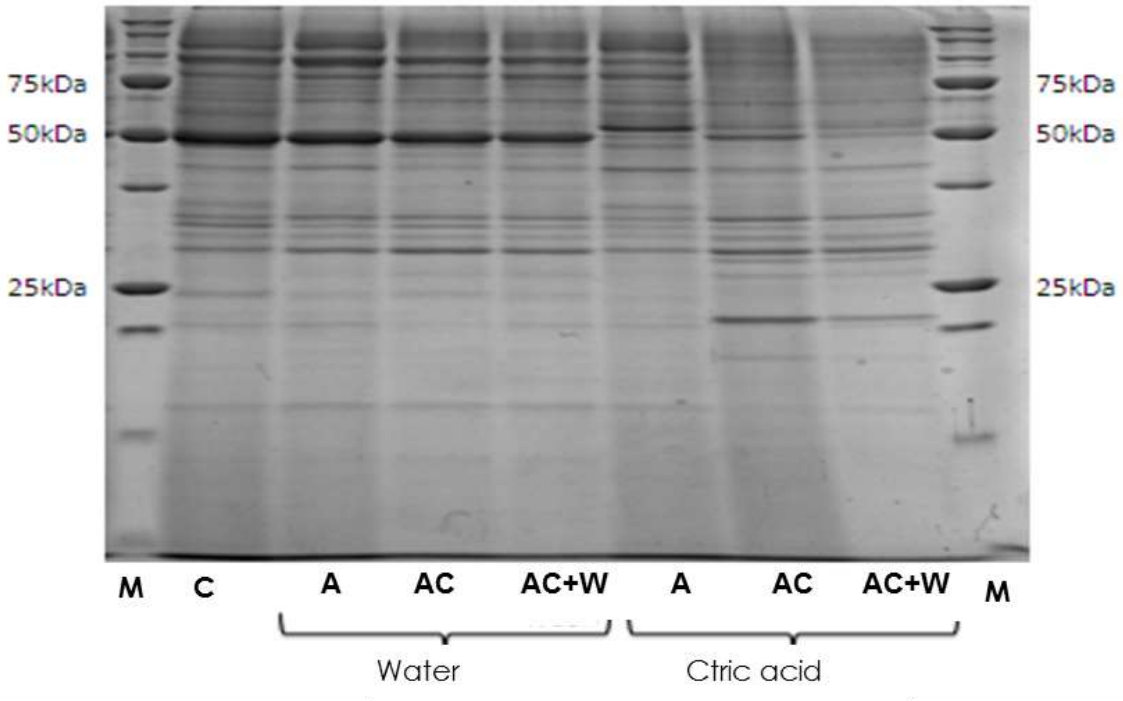

Fig. 2. SDS-PAGE of extracellular proteins produced in wheat bran extract medium. M: marker, C: Control, AC: AC medium, AC+W: AC wash medium. Water: washed by water. Citric acid: washed by citric acid solution.

TABLE 1. Comparison of extracellular protein contents and enzyme activity.

First step treatment solution	Processing step	Before incubation pH	After incubation pH	Protein μg/ml	α-Amylase mU/μg	Diastatic mU/μg	Xylanase (mU/μg)	Xylosidase (mU/μg)
Control		6.0	8.2	969	30.0	0.20	1.2	0.01
water	A	5.6	8.3	598	21.9	0.04	2.8	0.02
water	AC	6.7	7.8	410	24.1	0.1	4.6	0.02
water	AC+wash	6.9	7.7	345	13.5	0.0	3.4	0.02
citric acid	A	3.4	8.0	817	0.8	0.1	1.5	0.01
citric acid	AC	4.4	7.5	114	1.9	0.1	3.3	0.06
citric acid	AC+wash	5.6	7.0	118	0.5	0.0	2.2	0.07

TABLE 2. Identification of secreted proteins after AC+Wash treatment (Fig. 3B).

Gene ID	Gene name	spot
AO90120000068	catalase	15
AO90103000423	beta-1,4-xylanase	21, 35
AO90001000208	beta-1,4-xylanase	44, 50, 51, 53, 54, 55
AO90009000356	beta-glucosidase-related glycosidases	1, 2, 14,
AO90701000274	beta-glucosidase-related glycosidases	3, 4, 12
AO90012000135	beta-glucosidase-related glycosidases	13
DPP5_ASPOR	dipeptidyl-peptidase 5 precursor	8
AO90003001036	subtilisin-related protease/vacuolar protea	47
AO90003000923	predicted endo-1,3-beta-glucanase	20
AO90005000639	beta-N-acetylhexosaminidase	22
AO90003000476	mannosyl-oligosaccharide alpha-1,2-mannosidase and related glycosyl hydrolases	23
AO90003001062	manganese superoxide dismutase	42
AO90023000151	alpha-D-galactosidase (melibiase)	9
AO90012000445	beta-galactosidase	16
AO90003001017	predicted protein	5, 6
AO90020000289	predicted protein	11
AO90020000207	predicted protein	24
AO90003000990	predicted protein	31, 32
AO90020000213	predicted protein	34
AO90026000003	predicted protein	36
AO90120000026	predicted protein	45, 46

Analysis of two-dimensional electrophoresis

Proteomics was conducted using two-dimensional electrophoresis. Comparative proteome analysis of proteins secreted from *A. oryzae* RIB40 grown in standard wheat bran medium in which starch had not been removed is shown in Fig. 3A and wheat bran medium in which starch had been

removed by citric acid plus AC+Wash is shown in Fig. 3B. Spots surrounded by a circle are α-amylase, spots surrounded by a triangle are β-xylanase (Fig. 3A, Table 2). Spots surrounded by a square were only observed when starch was removed using citric acid plus AC+Wash. α-Amylase spots were not observed in the citric acid plus AC+Wash (Fig. 3). β-1,4-Xylanase was the most abundant protein. Three new β-glucosidases were discovered, in addition, endo-1,3-β-glucanase and β-galactosidase of the cellulase family were found. Glucose is required for *A. oryzae* to grow and if starch is removed, the glucose must be produced from a different source. Glucanase was highly expressed and was considered to have generated glucose. In the wheat bran culture medium in which starch was removed, α-amylase was not detected but many xylanases were. Two new β-glucanases were discovered and new predicted proteins were observed (Table 3). Exo-arabinanase, endo-1,4-β-xylanase A, glucan 1,3-β-glucosidase A were discovered as a new enzyme,

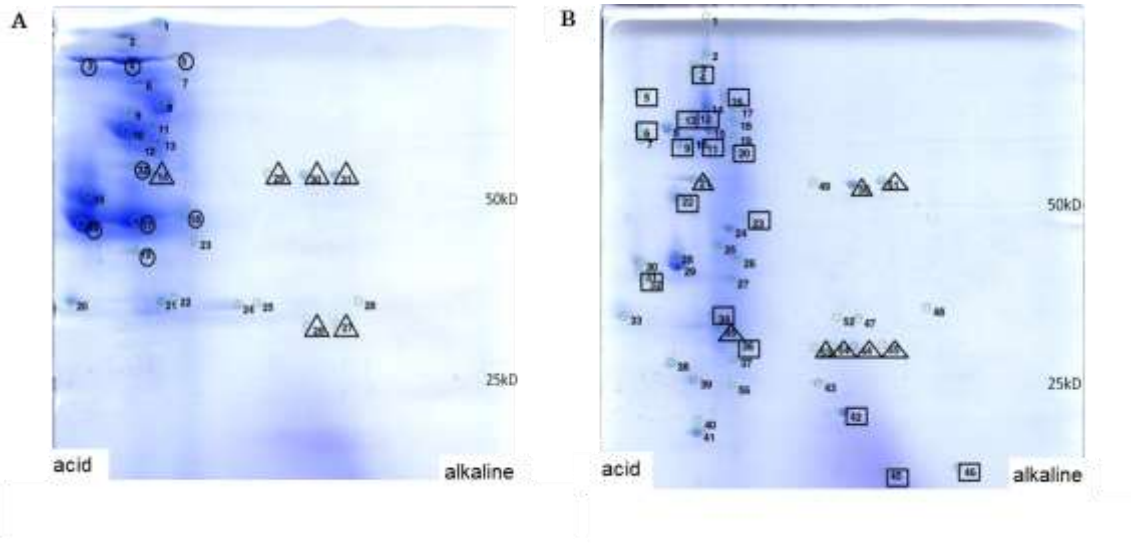

Fig. 3. Comparative proteome analysis of secreted proteins using control wheat bran medium (without treatment) (A) and AC+Wash with citric acid wheat bran medium (B) . Sequential differential display of secreted proteins from *A. oryzae* . ○: α-amylase, △: beta-1,4-xylanase, □: The spot which is not seen as (A)

TABLE 3. BLAST analysis of the protein spots(Fig. 2B).

Spot No.	Fungus	Gene name
31,32	*Aspergillus oryzae*	glucan 1,3-beta-glucosidase A
	Aspergillus flavus	probable glucan 1,3-beta-glucosidase A
5,6	*Aspergillus oryzae*	predicted protein
	Aspergillus flavus	BNR/Asp-box repeat domain protein
	Penicillium chrysogenum	exo-arabinanase
45,46	*Aspergillus oryzae*	endo-1,4-beta-xylanase A
	Aspergillus flavus	probable endo-1,4-beta-xylanase A
11	*Aspergillus oryzae*	predicted protein
	Aspergillus oryzae	glutaminase A
	Aspergillus flavus	glutaminase GtaA
34	*Aspergillus oryzae*	predicted protein
	Aspergillus fumigatus	putative uncharacterized protein
36	*Aspergillus oryzae*	predicted protein
	Aspergillus flavus	oxidocyclase
	Aspergillus flavus	FAD dependent oxidoredutase

when *A. oryzae* RIB40 was cultivated wheat bran without starch. It is suggested that newly observed proteins are induced when α-amylase is decreased, in particular, enzymes from the cellulase or hemicellulase families.

DNA microarray

RNA was extracted from *A. oryzae* RIB40 after treatment with AC+Wash plus citric acid and the control sample in which starch was not removed. DNA microarray was then performed.

The number of secreted cellulose-related genes decreased in the starch removed medium, although the cellulase activity were high. The expression level of β-1,4-xylanase was increased 27-fold and the expression levels of three newly identified endo-glucanases were 73, 70, and 10-fold higher than their expression in starch-containing medium. The expression level of β-xylosidase increased 3 fold. The genes in which the expression levels increased were considered to participate in solubilization of hemicellulose rather than the starch of wheat bran, cellulose, and lignin.

TABLE 4. Number of specific genes identified, using DNA microarray, from *A. oryzae* RIB40 when grown in either treated or untreated wheat bran medium.

	Wheat bran medium	Wheat bran without starch medium
hemicellulose gene	2	2
cellulose gene	10	7
previously-identified gene	21	13
unknown function gene	3722	1129
total	3757	1158

TABLE 5. Hemicellulase and cellulose genes in which significant variation in expression was observed between treated and untreated wheat bran medium.

Gene Name	Fold difference in expression
beta-1,4-xylanase	27
beta-xylosidase	3
endo-glucanase	73
endo-glucanase	70
endo-glucanase	10
beta-glucanase related glycosidase	3
alpha-glucosidase family31of glycosil hydrolase	38
beta-glucosidsse related glycosidase	5
glucsosidase I	2

Discussion

Removal of starch from wheat bran medium was investigated. We suggest citric acid is an effective method of starch removal from wheat bran: Autoclave treatment with citric acid and wash with hot water sufficiently removed starch such that α-amylase of *A. oryzae* RIB40 was not induced.

The specific activities of xylanase and xylosidase were increased after starch removal. It was considered that the specific activities increased because the nutrient source was altered; hemicellulose, cellulose, and lignin, basic components of wheat bran, were utilized instead of starch and we suggest the activities of xylanase and xylosidase

were increased because *A. oryzae* produced xylan as a nutrient source. Induction of xylanase and xylosidase, without induction of enzymes in the amylase family, can be achieved in *A. oryzae* RIB40 grown in treated wheat bran culture medium.

In addition, we identified β-glucosidase and endo-1,3-β-glucanase using two-dimensional electrophoresis and proteomics.

DNA microarray analysis of *A. oryzae* RIB40 grown in culture medium in which starch had been removed identified three new endo-glucanases and one β-xylosidase.

We suggest it would be possible to use the same solutions here for biomass decomposition and the altered gene expression could be useful in bioethanol production.

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