Molecular Cloning and Overexpression of fleA Gene Encoding a Fucose-specific Lectin of Aspergillus oryzae

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A protein from the cell lysate of Aspergillus oryzae was purified by column chromatography immobilized with a ferrichrysin (Fcy), which is one of the siderophores of A. oryzae. It is produced only in an iron-deficient culture and its molecular weight is estimated as 35,000 by SDS-PAGE. Two internal amino acid sequences of the protein obtained by lysylendopeptidase digestion were analyzed. Molecular cloning shows that it encodes 310 putative amino acid residues separated by 4 introns and is designated as fleA. It shows approximately 26% similarity with the gene encoding a fucose-specific lectin of Aleuria aurantia (AAL). The gene was overexpressed under control of the melO promoter in a submerged culture of A. oryzae. The fleA gene product showed hemagglutination activity against rabbit erythrocytes. A hemagglutination inhibition assay of monosaccharides showed that this lectin specifically binds to L-fucose and weakly reacts with mannose and N-acetyl-neuraminic acid.

Key words: fucose-specific lectin; Aspergillus oryzae; fleA; melO promoter; ferrichrysin

An A. oryzae siderophore, ferrichrysin (Fcy, MW 799), is a cyclic peptide containing a tripeptide of N-acetyl-δ-hydroxyornithine, a dipeptide of serine, and a glycine chelated with iron ion.1 It is produced by A. oryzae for acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3 For acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3 For acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3 For acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3 For acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3 For acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3 For acquisition of iron ions in iron-limited environments. In Japanese sake fermentation, an A. oryzae strain produces much Fcy in solid-state culture (koji) and it is responsible for unfavorable red coloring of sake.2 Although removal of Fcy is one of the most important problems in the quality control of sake, Fcy is difficult to remove by conventional methods with the active carbon and tannic acid frequently used in food manufacture. A technique for removal of Fcy has been demanded in Japanese sake.3

Materials and Methods

Strains, plasmids, and culture media. An A. oryzae strain (OSI 1018) isolated in our laboratory was used for production of Fcy. An A. oryzae strain (OSI 1013) was used for FAP preparation and molecular cloning of genes. The A. oryzae sc and niaD mutant (M-NS4) was used throughout the transformation experiments.5 pSC1, a 6.3-kb plasmid carrying the 3.0-kb BamHI fragment of the A. nidulans sc gene (DDBJ X82541) in pUC118 (Takara Shuzo, Japan) was used as the marker. E. coli JM109 (Takara Shuzo, Japan) and the pUC plasmids were used for DNA manipulation. The submerged culture for Fcy production was done at 35°C for 96 h in 21 of Fcy medium (2% glucose, 5% rice grains polished up to 70%, 0.003% EDTA, pH 6.0) in a jar fermentor (1vvm, 200 rpm). The iron-limited submerged culture for protein preparation was done at 30°C for 96 h in 21 of medium (2% glucose, 0.57%

Abbreviations: Fcy, ferrichrysin; FAP, ferrichrysin-affinity protein; AAL, lectin of Aleuria aurantia; cds, coding sequence; NANA, N-acetyl-neuraminic acid

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asparagine, 0.08% K$_2$HPO$_4$, 0.1% MgSO$_4$, 0.04% CaCl$_2$, pH 6.0) in a jar fermentor (1vvm, 200 rpm) and iron-containing submerged culture was done at 30°C for 96 h in 2 l of medium (2% glucose, 0.57% asparagine, 0.08% K$_2$HPO$_4$, 0.1% MgSO$_4$, 0.04% CaCl$_2$, 0.001% FeSO$_4$, pH 6.0) in a jar fermentor (1vvm, 200 rpm). The modified Czapek-Dox medium for protein overproduction under control of the melO promoter was 0.3% NaNO$_3$, 0.2% KCl, 0.1% KH$_2$PO$_4$, 0.05% MgSO$_4$·7H$_2$O, 0.002% FeSO$_4$·7H$_2$O together with 6% glucose, pH 6.0.

Purification of Fcy. The A. oryzae O-1018 strain was grown in the submerged culture for Fcy production. The 81 of culture broth contained 40 ppm of Fcy, and it was saturated with 100 ppm FeCl$_3$ solution. About 400 ml of Amberlite XAD-2 (Amersham Pharmacia Biotech, UK) was added, and a crude Fcy fraction was eluted with 2 l of 100 mM Tris-HCl buffer (pH 9.0), then 50 μl of 100 mM Tris-HCl buffer (pH 9.0) and 2.5 μg of lysylendopeptidase (Achromobacter protease I, Wako Pure Chemicals, Japan) was added. The reaction mixture was incubated at 37°C for 8 h. The reaction mixture was separated by reverse phase HPLC on μBONDASPHERE C-8 100 Å (Waters Co., USA) using a linear gradient of acetonitrile 0 to 100% at a rate of 1% per min. The peaks of peptide fragments were sequenced on a gas-phase protein sequencer (Applied Biosystems, MODEL 470A, USA).

Plaque hybridization. The degenerate oligonucleotide probe (5'-GAYAAYACIATICARGARTAYA-TGTGGAAYGGIGAYGG-3' containing deoxyinosine (I) and IUB code of mixed oligonucleotides) was synthesized based on the partial amino acid sequence (DNTIQEYMWNGDG). The probe was labeled with fluorescent dCTP using Gene Images 3'-oligolabeling system (Amersham Pharmacia Biotech, UK). 3000 plaques of lambda EMBL3 A. oryzae genomic library was transferred onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, UK) and were screened with the labeled probe. Hybridization was done at 50°C and detection was done with the Gene Images CDP-star detection system (Amersham Pharmacia Biotech, UK).

DNA sequencing. DNA sequencing was done using a BigDye-terminator kit and Genetic Analyser 310 (Applied Biosystems, USA).

Transformation and genomic Southern blot analysis. Transformation of E. coli was done by the method of Hanahan. The transformation of A. oryzae was done by the method of Iimura et al. The genomic DNA was prepared by the method of Tsuchiya et al. Gene Images labeling and a CDP-star detection system (Amersham Pharmacia Biotech, UK) was used for Southern blot analysis.

Polymerase chain reaction (PCR). PCR amplification was done on 100 ng of the template DNA in 100 μl of a reaction mixture containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl$_2$, 250 μM of each dNTP mixture, 100 pmol of each primer, and 2.5 U of Ex Taq DNA polymerase (Takara Shuzo, Japan). Thirty PCR cycles were used; denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min.

Expression of flbA gene under control of melO promoter in A. oryzae. Combined PCRs (Higuchi) were done to construct the fusion genes of the melO promoter, flbA cds, and the flbA terminator. The melO promoter (−1173 to −1) was amplified by PCR with the upper primer M1 (5’-AACTGCAG-3’).
GCTGCCTTGCTCAAATCG-3′; the synthesized PstI site underlined), and the lower primer M2 (5′-AGGAGTAGACATCTGGAACCAAAGTAATCAGAAG-3′; the underlined sequences being capable of annealing with those in the F1 primer). The fleA open reading frame and terminator (each 1151 and 479 bp downstream from the start codon) also were amplified by PCR with the upper primer F1 (5′-CTTTGGTTCACAAATGTCTACTCTGTCGC-3′; the underlined sequences being capable of annealing with those in the M2 primer) and the lower primer F2 (5′-AATTCCGTCTTCTCTATGGAACTCCGTT-3′; the synthesized PstI site underlined). Each mixed PCR product was given to a 2nd PCR amplification with the M1 and F2 primers. The PCR products (2.8 kb) obtained were digested with PstI and sub-cloned into pSC1, giving pSMF1 (Fig. 3A). Amplification of the DNA fragment was confirmed by nucleotide sequencing analysis. pSMF1 was used to transform into A. oryzae M-NS4 strain by the method of Yamada et al., then the transformant was analyzed by Southern blotting. The transformant selected was cultured in 100 ml of modified Czapek-Dox medium at 30°C for 7 days. After collection of the mycelia, a cell-free extract was prepared by disruption with liquid N2 followed by extraction with 10 mM KPB (pH 7.0).

Hemagglutination and inhibition assay. The titration of lectin was done by sequentially diluting the sample with PBS, then mixed with an equal volume (25 μl) of 2% rabbit erythrocytes suspended in PBS. In inhibition assays, the lectin (1.4 μg in 25 μl of PBS) was incubated with sugars at room temperature for 1 h, then assayed for the residual hemagglutinating activity. The minimum concentration of sugar that completely inhibited the hemagglutination was found.

Results

Column chromatography immobilized with Fcy

For screening of a novel Fcy-specific binder, Fcy was coupled with 10 ml of epoxy-activated sepharose 6B under alkaline conditions. The coupling efficiency was more than 95% (data not shown). A Fcy-specific binder is expected to exist as an intracellular protein of A. oryzae. An A. oryzae strain was cultured in two different cultures with or without ferric ion, and a cell-free extract of each was chromatographed on a column immobilized with Fcy. A washing step was done with 10 mM KPB (pH 7.0) and elution was done in order of high salt (1 M NaCl), acid (0.5 M citrate-1 M NaH2PO4, pH 3.0), and detergent (1% SDS). Figure 1 showed SDS-PAGE analysis of each eluate. No specific proteins were detected in each eluate of the cell-free extract from A. oryzae cultured in the medium containing ferric ion. However, a single band was detected in acid and detergent eluates from A. oryzae cultured without ferric ion, suggesting that it specifically bound to the Fcy column. FAP was not prepared from a cell-free extract of A. oryzae cultured with ferric ion. The molecular weight of the FAP subunit showed 35,000. FAP was completely purified by elution with detergent without further purification.

Analysis of partial amino acid sequences

The purified FAP was digested by lysylendopeptidase for amino acid sequencing. The digests were separated by reverse phase HPLC, and several partial fragments were collected. Two partial amino acid sequences were analyzed; P1: TDNTIQEYMWN-GDWK and P2: EGTNLGVALPGTGIGVT. The N-terminal sequence of FAP could not be identified because of its N-terminal blocking (data not shown).

Molecular cloning and DNA sequence of fleA gene

The degenerate oligonucleotide probe (5′-GAYAAYACIATCARGARTAYATGGAAYGGIGAYGG-3′) was synthesized based on the amino acid sequence of an internal peptide, P1. The fluorescent dCTP-labeled probe was used for screening of a lambda EMBL3 A. oryzae genomic library. After
Fig. 2. Nucleotide and Deduced Amino Acid Sequences of \( f\)leA.

Four regions described in small letters show introns. The amino acid sequences found by amino acid sequencing are underlined. The putative binding sites of \( sreA\)-like gene product is located in “470 and “193 bp described in bold and italic letters. The sequence data is submitted to DDBJ/EMBL/GenBank under No. AB072379.
Fig. 3. Overexpression of \( \text{fleA} \) under the \( \text{melO} \) Promoter Control in \( A. \text{oryzae} \).

(A) Construction of the \( \text{fleA} \) overexpression plasmid (pSMF1). Methods for construction is described in Materials and Methods. (B) SDS-PAGE analysis of the cell-free extract from the transformant harboring pSMF1 (lane T). Control shows the cell-free extract from the transformant harboring pSC1 (lane C). Ten \( \mu \text{g} \) of protein was put in each lane. The arrow shows the band corresponding to FAP.

Discussion

This is the first report on isolation of a lectin by column chromatography immobilized with a siderophore. The first idea is addressed in isolating a protein which had an affinity with the \( A. \text{oryzae} \) siderophore Fcy,\(^1\) which has been difficult to be removed in the sake fermentation. We isolated the Fcy affinity protein (FAP) from a cell-free extract of \( A. \text{oryzae} \) cultured in the iron-deficient medium, followed by molecular cloning of FAP. Unexpectedly, this gene product shows 26\% similarity with a fucose-specific lectin of \( \text{Aleuria aurantia} \) (AAL).\(^3\)

This protein is expressed in the iron-deficient culture, but is repressed in the culture containing iron. The 5'-noncoding region of \( \text{fleA} \) contains two GGA-TA sequences at −470 and −193 bp, consensus DNA binding sites of GATA family transcription factor \( \text{sreA} \) of \( \text{Aspergillus nidulans} \).\(^{15}\) The \( \text{sreA} \) is involved in siderophore biosynthesis for iron acquisition and negatively regulates siderophore biosynthetic genes. The \( \text{sreA} \) is reported to regulate the genes involved in iron homeostasis in addition to siderophore biosynthesis. These findings suggest that the \( \text{sreA} \)-like transcription factor would regulate expression of \( \text{fleA} \) in \( A. \text{oryzae} \). The biological function of fungal fucolectin encoded by \( \text{fleA} \) remains unclear. The major pathogenic fungus \( \text{Candida albicans} \) is known to express fucolectins for adhesion to host cells in the infection stage.\(^{16}\) This led us to the hypothesis that the \( A. \text{oryzae} \) lectin could be involved in adhesion to carriers under iron-deficient conditions such as solid-state culture, because \( A. \text{oryzae} \) partially secreted the lectin as an extracellular protein in solid-state culture (data not shown).

The lectin specifically bound to the Fcy-immobilized column, but Fcy showed no hemagglutination inhibition activity of the lectin (Fig. 4B). The lectin was not at all trapped by a blank column containing only the spacer without Fcy (data not shown). Therefore the lectin could recognize the partial structure of Fcy immobilized on the sepharose 6B column. More detailed binding assays may reveal an interaction between the lectin and Fcy.

Expression of \( \text{fleA} \) under the \( \text{melO} \) promoter control in \( A. \text{oryzae} \) led to accumulation as the most abundant protein in total intracellular protein. \( A. \text{oryzae} \) is known to be an excellent host for extracellular secretion of genetically modified proteins,\(^{17}\) but there is little known about overproduction of genetically modified proteins in the intracellular protein of \( A. \text{oryzae} \). There is great interest in accumulation of the lectin as the most abundant protein in total in-
tracellular protein of *A. oryzae* without proteolysis. Overexpression of the lectin in *A. oryzae* caused no different phenotypes from the wild strain. Overexpression by the *melO* promoter enables *A. oryzae* to secrete 3.3 g/l-broth of glucoamylase and to accumulate the lectin as the most abundant protein in total intracellular protein.

Investigation of the sugar-binding specificity of the lectin showed that only L-fucose inhibited the hemagglutination activity of the recombinant *fleA* gene product, and that NANA and D-mannose were very weak inhibitors. L-fucose is an approximately 50-fold stronger binder than NANA and D-mannose. The specificity of lectin for L-fucose is equivalent to AAL. The oligosaccharides binding study shows that AAL is highly specific for the oligosaccharides containing α-1, 6 fucosyl residues. Investigations of the specificity of the lectin for fucosyl oligosaccharides are currently in progress.

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**References**


