Note

Functional Expression in Aspergillus oryzae of p15, a Protein with Potent Neurite-inducing Activity in PC12 Cells

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Received September 17, 2001; Accepted November 5, 2001

We previously reported that a fungal protein, p15, induces neurite outgrowth and differentiation of rat pheochromocytoma PC12 cells through the activation of the Ca\(^{2+}\) signaling pathway. We report here the secretory production of p15 in Aspergillus oryzae. Analysis of culture supernatant of A. oryzae transformed with the gene encoding the p15 precursor tagged with a hemagglutinin (HA) epitope demonstrated that the transformant secreted a protein with an apparent molecular mass of 17.5 kDa, which is a little larger than the expected size of mature p15-HA. By heat denaturation and ion exchange chromatography, p15-HA was easily purified from the culture supernatant with sufficient abundance. Although purified p15-HA was less active than the native p15 obtained from the culture broth of a producing fungal strain, it had neurite-inducing activity in PC12 cells in a dose-dependent manner, providing a system to study the action mechanism of p15.

Key words: Aspergillus oryzae; neurite outgrowth; PC12; neuronal differentiation; fungal 15-kDa protein

Filamentous fungi, especially those in the genus Aspergillus such as A. oryzae, have been successfully used for the purpose of heterologous protein expression as well as in the fermentation industry. With its high ability of secreting proteins into the culture media, A. oryzae has served as an efficient system for foreign protein production and secretion, including mammalian secretory proteins. In addition, recent development of an expression vector system using an improved A. oryzae glucoamylase promoter has further facilitated extensive use of A. oryzae in protein production for various aims.

We previously identified and purified a 15-kDa protein, p15, from the culture broth of a fungus, genus Helicosporium, which potently stimulates neurite formation and neuronal differentiation of rat pheochromocytoma PC12 cells. At concentrations as low as 1 nm, p15 induces neurites and expression of neurofilament-M protein in PC12 cells. These effects of p15 have been shown to be dependent on the activation of voltage-dependent L-type Ca\(^{2+}\) channels and subsequent activation of the Src-Ras-MAP kinase cascade, since PC12 cells failed to respond to p15 in the presence of specific inhibitors of these cellular pathways. Furthermore, we cloned the gene encoding p15 precursor from the genomic DNA of a producing fungal strain. The predicted primary structure of the p15 precursor suggested that, compared to the mature region with neurite-inducing activity, it has additional sequences at the amino- and carboxy-ends. In this report, we describe secretory production of p15 by introducing the p15 precursor gene into A. oryzae, the expression of which was driven by the improved glucoamylase promoter. Subsequent purification and examination of activity demonstrated that the p15 produced in A. oryzae was functional, albeit lower in activity than native p15.

Construction of a plasmid and transformation of A. oryzae for the expression of hemagglutinin epitope-tagged p15. In our previous studies, we reported the identification and purification of p15 from the culture supernatants of the producing fungal strain. Subsequent cloning of the genomic DNA encoding p15 and nucleotide sequence analysis suggested that p15 is produced as a precursor with amino- and carboxy-terminal extensions. Within the amino terminal extension sequence, a putative signal peptide was found, which is consistent with our observation that p15 is a secretory protein. In addition, the p15 precursor has amino- and carboxy-terminal extension sequences 13 and 20 amino acids in length, respectively, which would presumably be required for proper folding of the mature region. With an expectation that these amino- and carboxy-terminal extra sequences will function and be correctly processed in the heterologous expression system in A. oryzae, we introduced full-length p15 precursor gene into A. oryzae. We also expected that an 81-bp intron in the p15 precursor gene would be correctly spliced out in A. oryzae. In addition, to
Fig. 1. Schematic Representation of the Plasmid for the Expression of p15-HA in *A. oryzae*.

The HA epitope was inserted between the mature and the carboxy-terminal extension sequence of p15 by PCR as described in the text. The amino acid sequences of the HA epitope and the flanking region of p15 are shown at the bottom along with the nucleotide sequences of the primers used. In the middle of the figure, the schematic structure of p15-HA is shown as follows: amino- and carboxy-terminal extensions in the p15 precursor, gray boxes; mature region of p15, black box; HA tag, hatched box; intron, white box. The DNA fragment encoding p15-HA was inserted into pNGA142 between the improved glucoamylase promoter (P \textsc{gla}A\textsubscript{142}) and \(\alpha\)-glucosidase terminator (T \textsc{agd}A\textsubscript{10}), generating pNH88.

Expression of p15 in *A. oryzae* facilitate detection of p15 produced, a hemagglutinin (HA) tag was inserted between the mature and carboxy-terminal extension sequence of p15 by a PCR-based method\(^7\) as shown in Fig. 1. Two primers, p15-HA-S and p15-HA-AS, were designed in inverted tail-to-tail directions to amplify the plasmid pB8 in which the 0.8-kb DNA fragment encoding the p15 precursor was inserted between Eco\textsc{R}V and Eco\textsc{RI} sites of pBluescriptII SK\textsuperscript{+}. The sequences of primers were: p15-HA-S, 5\textsuperscript{'}-TCCGGATTACGCTaagaagagggcagcagaa-3\textsuperscript{'}; p15-HA-AS, 5\textsuperscript{'}-ACATCGTATGGGTAactaccgaatgtcttaacagc-3\textsuperscript{'}.

The PCR reaction of 94°C, 5 min, 25 cycles at 94°C, 1 min, 50°C, 1 min and 72°C, 4 min, then 72°C, 7 min extension was done in 100 \(\mu\)l of reaction mixture as instructed by the Expand High Fidelity PCR System (Roche Diagnostics). The PCR product was purified by a GENE CLEAN II Kit (Bio 101 Inc.) from a portion of the agarose gel. The purified DNA was self-ligated using a DNA Ligation Kit ver.2 (TaKaRa), followed by use for transformation of *E. coli* DH5\(\alpha\) by the method of Hanahan.\(^8\) The plasmid thus prepared was named pBH88. The DNA sequence was analyzed with a Shimadzu DSQ-2000L DNA sequencer, which confirmed that the HA epitope (YPYDVPDYA) was correctly inserted between the carboxy-terminal sequence of mature p15 (AVKTFGS) and the carboxy-terminal extension of p15 precursor (KKRAAE...).

The plasmid for expression in *A. oryzae*, pNH88, was constructed by inserting a DNA fragment from pBH88 encoding p15-HA into the multiple cloning site of pNGA142, which contains an expression cassette consisting of an improved \(\textsc{gla}A\) promoter (P\textsc{gla}A\textsubscript{142}) and an \(\alpha\)-glucosidase terminator (T\textsc{agd}A\textsubscript{10}).\(^9\) *A. oryzae* niaD300 strain (niaD\textsuperscript{-}) was transformed with pNH88 as described by Gomi *et al.*\(^10\) Transformants were selected three times for nitrogen auxotrophy on Czapek-Dox (CD) medium (0.3% NaNO\textsubscript{3}, 0.2% KCl, 0.1% KH\textsubscript{2}PO\textsubscript{4}, 0.05% MgSO\textsubscript{4} \(\cdot\) 7H\textsubscript{2}O, 0.002% FeSO\textsubscript{4} \(\cdot\) 7H\textsubscript{2}O, 2% glucose, pH 5.5), which resulted in the isolation of four transformants (NH881, NH882, NH883, and NH884). They were grown in the liquid DPY production medi-
um (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O, pH 5.5) by shaking at 120 rpm for 3 days at 30°C to drive expression of the cloned gene from the glaA promoter. The cells were removed by centrifugation (10,000 × g for 10 min at room temperature) and the supernatants were collected. Approximately 15 microliters of culture supernatants were analyzed for the presence of p15-HA. SDS-PAGE was done on 15% acrylamide gels under reducing conditions, and for Western blotting, proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Nihon Eido, Tokyo). The blotted membranes were blocked with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). The membranes were then probed with mouse anti-HA antibody (1: 500 dilution; Roche Molecular Biochemicals) for 1 h, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (1: 500 dilution; Vector), and developed using 4-chloro-1-naphthol. One of the four transformants, NH881, was found to secrete a significant amount of p15-HA (Fig. 2A and 2B). No corresponding band was detected in the culture supernatant of the vector-transformed strain, and the pelleted mycelial fractions did not contain p15-HA (data not shown). The apparent molecular mass of the band was approximately 17.5 k, which was a little larger than the calculated molecular mass of mature p15 plus HA tag (14.4 k). There is no consensus sequence for N-glycosylation in the amino acid sequence of mature p15. We speculate that retarded electrophoretic mobility is due to the presence of an amino-terminal prosequence (13 amino acids long) and the biochemical nature of p15; the calculated molecular mass of the amino-terminal prosequence is approximately 1.4 k, and in SDS-PAGE p15 migrates as if larger than the authentic size by 1.7 k, which, together with the mature region of p15-HA, would sum up to the net apparent molecular mass of 17.5 k. Productivity of p15-HA was estimated to be about 25 mg per liter of culture broth, which is comparable or slightly better than the production of hexahistidine-tagged p15 in E. coli. The productivity of p15-HA was estimated by comparison with bovine serum albumin as a standard on Coomassie Brilliant Blue (CBB)-stained gels.

Production and purification of p15-HA. Next we purified p15-HA from the culture supernatant of NH881. The supernatant of transformant NH881 was concentrated up to 10-fold by lyophilization, and was then boiled for 10 min and centrifuged (100,000 × g for 30 min at 4°C) to remove contaminating proteins. As expected from the heat-resistant nature of native p15, p15-HA was resistant to boiling for 10 min (data not shown). The heat-resistant fraction was dialyzed against distilled water at 4°C overnight and was further purified by DEAE-cellulose column chromatography (DE52, Whatman; Fig. 3). Proteins were eluted in 50 mM Tris-HCl (pH 7.4) containing a stepwise gradient of 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, and 250 mM NaCl. Elution of p15-HA from the column was observed at 40–60 mM NaCl in which p15-HA was purified to more than 80% purity. The samples of lane 6 and 8 (Fig. 3) were dialyzed twice against 20 mM sodium phosphate (pH 7.1–7.4) for 12 h at 4°C and were stored at −20°C until use. Approximately 0.6 mg of purified protein was obtained from the 40 ml of the culture broth, with the purification yield of about 60%. Biological activity of p15-HA. The activities of the culture supernatant of NH881 as well as purified p15-HA were examined by neurite-induction assay in PC12 cells. PC12 cells were maintained in DMEM supplemented with 5% horse serum and 5% fetal calf serum. The cells were passaged every 3–4 days and kept at 37°C in 10% CO₂ under humidified air. PC12 cells were seeded in the growth medium at 4.5 × 10⁴ cells/cm² in collagen type I (Becton Dickinson)-coated 24-well culture plates and allowed to grow for 24 h. The reagents were added, and neurite outgrowth was measured after 48 h. To measure neurite outgrowth, two random photographs were taken per well, cells bearing processes longer than the cell diameter being considered positive. PC12 cells treated with the culture supernatant of NH881 extended neurites, while the control cells treated without or with
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the culture supernatant of the vector-transformed strain did not (Fig. 4A and data not shown). The purified p15-HA described above also induced neurites in PC12 cells. The specific activity of p15-HA in the neurite-extension assay was examined and compared with that of native p15 that was purified from the acetone-extracted culture supernatant of the producing fungal strain. As shown in Fig. 4B, the specific activity of p15-HA was lower than that of native p15, but the neurites were indeed induced in a dose-dependent manner of p15-HA; at 10 and 100 nM of p15-HA, 21 and 34% of PC12 cells extended neurites, respectively, while a similar extent of neuritogenesis was observed with 1 nM native p15. It is highly probable that retention of amino-terminal prosequence, which is assumed from the retarded electrophoretic mobility of p15-HA, as well as the presence of the HA-tag at the carboxy-terminus are the main causes of the lower specific activity of p15-HA.

In this study we produced functional p15 in the culture supernatant of A. oryzae transformed with p15 gene tagged with the HA epitope. The protein was purified by a simple strategy consisting of heat-treatment and ion-exchange chromatography. Although the productivity of p15-HA was at the similar level or higher than that reported for the heterologous expression of mammalian proteins in A. oryzae,11,12 it was low for the expression of a foreign protein of fungal origin. It has been reported that the titration of transcription factor(s) that competes for binding to the upstream regulatory element of exogenously introduced, tandemly-repeated α-glucosidase promoter and endogenous α-amylase promoter occurs, especially when the multiple copies of expression vector was introduced. In this study, we observed little, if any, reduction in the amount of α-amylase in the culture media of NH881 and other
transformants compared with the parental strain (Fig. 2A), suggesting that NH881 contains only single or low copies of the expression plasmid pNH88. We also cultured *A. oryzae* transformants in wheat bran solid-state media since growing *Aspergillus* in the solid state culture often improves the productivity of commercial enzymes. The production of p15, however, was not increased in either a water extract of wheat bran culture nor a cell extract fraction (data not shown). Further adjustment of culture conditions as well as isolation of transformants containing multiple copies of the p15 gene is needed to improve the production level of p15, which would allow study on the structure-function relationship of p15 mutants created by site-directed mutagenesis.

**Acknowledgments**

This work was supported by a Grant-in-Aid for Scientific Research (No. 12460041 to K. K. and No. 12760048 to M. A.) from the Ministry of Education, Science, Sports, and Culture of Japan, and by a grant from the Fujisawa Foundation.

**References**


