Appl Microbiol Biotechnol (1991) 35:611–614
0175759891001908

Applied and Microbiology Biotechnology

Positive identification of a lambda gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification*

E. J. Mullaney[1], D. M. Gibson[2], and A. H. J. Ullah[1]

[1] USDA, ARS, Southern Regional Research Center, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA
[2] USDA, ARS, North Atlantic Area, Cornell University, Tower Road, Ithaca, NY 14853, USA

Received 23 October 1990/Accepted 15 April 1991

Summary. As the initial step in a project to provide a more cost-effective source of the phytase enzyme, this paper reports on the use of a polyclonal antibody raised to phytase purified from an isolate of *Aspergillus niger* (*A. ficuum*) to screen an *A. niger* lambda gt11 expression library and the use of amino acid sequencing to identify a clone containing part of the fungal phytase gene. The described use of amino acid sequence fragments to verify the cloning of a gene has potential applications in other cloning projects.

Introduction

The extracellular enzyme, phytase, has previously been purified from an isolate of *Aspergillus niger* (Ullah and Gibson 1987). Phytase (myo-inositol hexaphosphate phosphohydrolyase, E.C. 3.1.3.8) degrades phytic acid, the primary storage form of phosphate in cereal grains, legumes, and oilseeds such as soy, which are principal components of animal feed. Phytic acid is an undesirable component in feeds for monogastric animals since the phosphate in phytic acid and essential minerals and protein ionically bound to it are nutritionally unavailable (Reddy et al. 1982). A cost-effective source of this enzyme has been sought for several years to enhance the value of soybean meal as an animal feed (Shieh and Ware 1968), eliminating the need for costly phosphate supplements in feeds. The phytase from *A. ficuum*, recently placed in synonymy with *A. niger*, has been used successfully as a feed additive in chicken meal unsupplemented with dicalcium phosphate (Nelson et al. 1971), although the cost of enzyme production at that time was not competitive with phosphate addition. The lack of a cost-effective source of this enzyme still blocks commercial development of this application today.

As part of a project to obtain increased levels of phytase production and increase the activity of the enzyme, the fungal phytase gene is now being cloned.

This paper reports on the utilization of a polyclonal antibody in the screening of an *A. niger* lambda gt11 expression library (Young and Davis 1985), and the successful isolation of a recombinant clone containing a 264-bp DNA sequence near the center of the fungal phytase gene. Positive identity of this clone was made possible by matching the amino acid sequence from the translation of its open reading frame region with the sequence obtained from the fungal phytase protein.

Materials and methods

Protein purification for primary structure. A. niger phytase and pH 6.0-optimum acid phosphatase were purified from the crude culture filtrate by sequential soft-gel chromatography as developed in this laboratory (Ullah and Gibson 1987; Ullah and Cummins 1988). The purified enzymes were derivatized by 4-vinylpyridine in the presence of dithiothreitol to alkylate cysteine by a published procedure (Thomsen and Bayne 1988) and then further purified by C8 (Synchropak, Lafayette, Ind., USA) reversed-phase HPLC. Purified pyridylethylated (PE) phytase was subjected to enzymatic cleavage with trypsin in the presence of 2.0 M urea (Tarr 1986). Peptide mapping and purification were obtained using a C18 (Vydac, Hersperia, Calif., USA) reversed-phase HPLC. Peptide sequencing was elucidated by a Porton PI 2090 on-line gas phase microsequencer using procedure 40 (Porton Instruments, Tarzana, Calif., USA).

Antibody production. Fungal phytase was purified as previously described (Ullah and Gibson 1987). Polyclonal antibody to fungal phytase was produced in rabbits using three subcutaneous injections of 0.5 mg each mixed in incomplete Freund's adjuvant by a commercial sera company (Pel-Freez, Royers, Al., USA). To generate affinity-purified antibody for immunoscreening analysis; polyclonal antisera from several bleeds were pooled. To eliminate cross-reactivity from *Escherichia coli* lysates, the antiserum was passed over an affinity column prepared from an *E. coli* lysate Y1090 column (5 Prime → 3 Prime, Inc., West Chester, Penn., USA). The flow-through was collected from a phosphate-buffered

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Offprint requests to: E. Mullaney

saline (PBS), pH 7.2, wash, and the column was regenerated following a 1 M NaCl rinse.

The *E. coli*-adsorbed antiserum was then subjected to affinity purification. Fungal phytase, purified as in Ullah and Gibson (1987), was coupled via cyanogen bromide activation to Sepharose 4B (Pharmacia, Uppsala, Sweden) at a concentration of 10 mg/ml using the manufacturer's instructions, and equilibrated with PBS, pH 7.2. Antiserum was passed over the column and rinsed with PBS until the absorbance at 280 nm (A_{280}) returned to baseline levels. Affinity-purified antiserum was removed with 0.2 M glycine, pH 2.3, and dialysed against PBS, pH 7.2, concentrated to 1 mg/ml, and frozen in 50-µl aliquots at −20° C until use.

Immunoblotting. Western blots were prepared according to standard protocol (Laemmli 1970). Briefly, standard 12% Laemmli gels were transferred to nitrocellulose (Bio-Rad, Richmond, Va., USA) or to Nitrocellulose Plus (MSI, Honeoye Falls, New York, NY, USA) using a BioTrans semi-dry electrophoretic transfer unit (Gelman, Ann Arbor, Mich., USA) with 48 mM TRIS, 39 mM glycine, 1.3 mM sodium dodecyl sulfate, and 20% methanol, pH 8.4, at 30 mA for 90 min. Rainbow markers (Amersham, Arlington Heights, Ill., USA) were used to determine molecular weights on Western blots.

Genomic DNA preparation. Total DNA was isolated from *A. niger* (SRRC 265) as described by Klich and Mullaney (1987) and the nuclear DNA was separated from mitochondrial DNA by CsCl/bisbenzimide density gradient centrifugation (Garber and Yoder 1983). One hundred micrograms of the nuclear DNA was then sheared with a 26-gauge needle until the major portion of the DNA was less than 7 kb in length. The sheared DNA was used to construct a lambda gt11 genomic expression library as described by Young and Davis (1985).

*Eco*RI for restriction enzyme analysis of the recombinant lambda gt11 clones was obtained from Pharmacia (Uppsala, Sweden) and Sequenase Version 2.0 (United States Biochemical Corporation, Cleveland, Ohio, USA) was used for DNA sequencing of the insert after it was subcloned into M13mp19.

Immunoscreening. E. coli-adsorbed, affinity-purified antisera was used for immunoscreening analysis (Young and Davis 1985). Dot blot analysis indicated that a titer of 1:10000 would distinguish approximately 1 ng of fungal phytase. Nitrocellulose filters were removed from cold plates using forceps and placed in a rinse solution of 0.25% gelatin in TTBS (50 mM TRIS, 500 mM NaCl, pH 7.2, 0.05% Tween-20) for approximately 1 min per filter. Filters were then transferred into a blocking solution of 2% gelatin in TTBS for 1 h at RT with gentle agitation, followed by transfer into a 1:10000 dilution of primary antisera in 1% gelatin in TTBS for 1–2 h with gentle agitation. Filters were then washed twice for 10 min with TTBS, then placed into 1% gelatin in TTBS containing a 1:3000 dilution of goat-anti-rabbit immunoglobulin G (IgG) alkaline phosphatase-labelled antisera for 1 h at RT with gentle agitation. Filters were then rinsed twice for 10 min with TTBS, followed by a one 10 min rinse in TBS (TRIS-buffered saline minus Tween-20). Color was developed with 30 mg nitroblue tetrazolium (1 ml in 70% dimethylformamide), 15 mg bichloroindole phosphate (1 ml in dimethylformamide) in 100 ml of 10 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8. Color development was stopped after 15 min by immersing filters for several changes in distilled water.

Results

Western blot analysis of antibody reactivity

Phytase and pH 6.0-optimum acid phosphatase were recognized via immunochemical staining using antibody raised to fungal phytase on Western blot (Fig. 1). Phytase had a typical diffused staining pattern indicative of its glycoprotein nature, migrating at approximately 80–100 kDa. The pH 6.0-optimum acid phosphatase, migrating at approximately 82 kDa, also stained with the antiserum, raising the possibility that there are conserved amino acid regions in common between phytase and pH 6.0-optimum acid phosphatase.

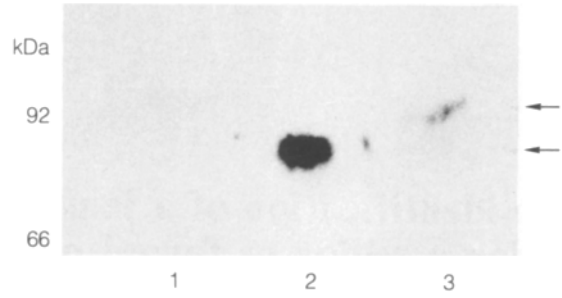

Fig. 1. Immunoblotting of *Aspergillus niger* phytase and pH 6.0-optimum acid phosphatase using anti-fungal phytase as the primary antiserum, and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate as the secondary antiserum. *Lane 1*, 0.5 µg phytase; *lane 2*, 1.0 µg pH 6.0-optimum acid phosphatase; *lane 3*, 1.0 µg phytase. *Arrows* pinpoint the position of bands in *lanes 2* and *3*

Tryptic peptide maps of phytase and pH 6.0-optimum acid phosphatase

Tryptic maps of purified phytase and pH 6.0 optimum acid phosphatase, as established by a C18 reversed-phase HPLC, are shown in Fig. 2A and 2B. The differences between the two maps implies a lack of sequence homology with respect to trypsin cleavage sites (Lys and Arg) and that these proteins are not identical.

Several of the fragments from the trypic digest of phytase were purified and subjected to N-terminal sequencing. The amino acid sequence of fragments 20, 30, 31 and 41 from this map all exactly correspond to different regions of this deduced sequence of 87 amino acids obtained from the A-3 clone (Fig. 3).

Immunoscreening of lambda gt11 library

Four positive isolates were retained after three cycles of immunoscreening of plaque lifts. DNA from all four of the recombinant clones were isolated, digested with the restriction enzyme *Eco*RI and subjected to electrophoresis. Results suggested that all four recombinants were identical, each having *Eco*RI inserts of approximately 270 bp of DNA.

One of the recombinants, A-3, was subcloned into the *Eco*RI site of M13 mp19, and the insert was sequenced. Figure 3 shows the 264-bp DNA sequence of the A-3 insert and the flanking *Eco*RI linkers. A translation of one of the three reading frames yielded a continuous open reading frame of 87 amino acids.

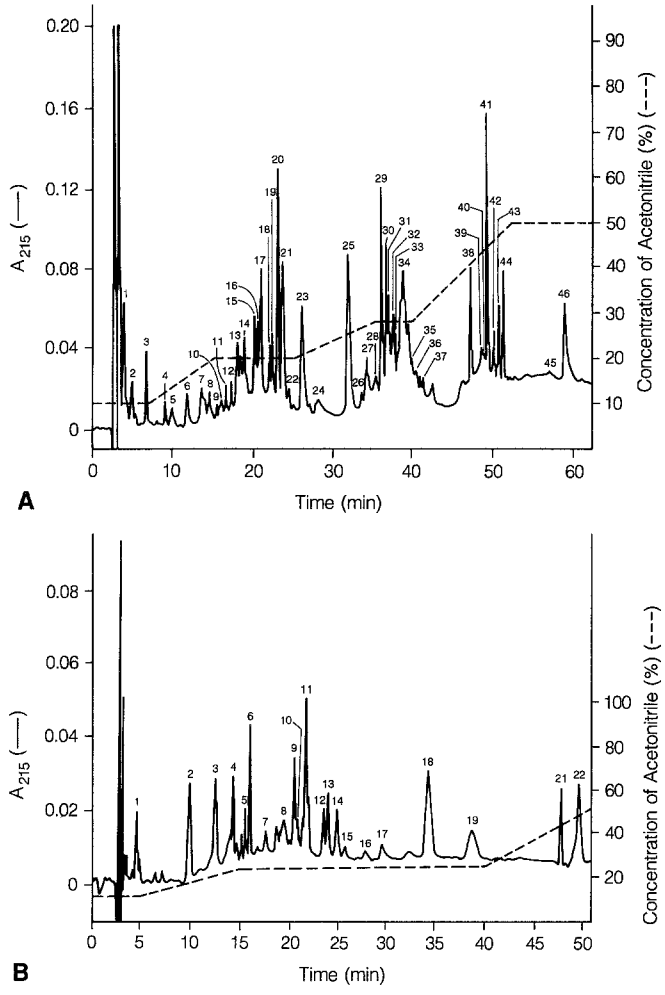

Fig. 2. Reversed-phase (C-18) HPLC peptide maps of tryptic digests of phytase (**A**) and pH 6.0-optimum acid phosphatase (**B**): A_{215}, absorbance at 215 nm

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GAATTCC CC GAT ACC GTC GAA GCC AAT TTC ACC GCC ACG TTC GTC CCC
           Asp Thr Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro

TCC ATT CGT CAA CGT CTG GAG AAC GAC CTA TCC GGT GTG ACT CTC ACA
Ser Ile Arg Gln Arg Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr
      [31] ..................................................

GAC ACA GAA GTG ACC TAC CTC ATG GAC ATG TGC TCC TTC GAC ACC ATC
Asp Thr Glu Val Thr Tyr Leu Met Asp Met Cyc Ser Phe Asp Thr Ile
.............    [30] ..................................

TCC ACC AGC ACC GTC GAC ACC AAG CTG TCC CCC TTC TGT GAC CTG TTC
Ser Thr Ser Thr Val Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe
..........              [41] ..........................

ACC CAT GAC GAA TGG ATC AAC TAC GAC TAC CTC CAG TCC TTG AAA AAG
Thr His Asp Glu Trp Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys
.................................................... [20]

TAT TAC GGC CAT GGT GCA GGT AAC CCG CTC G GGAATTC
Tyr Tyr Gly His Gly Ala Gly Asn Pro Leu
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Fig. 3. The 264 DNA base sequence of A-3 flanked by the *Eco*RI linkers (*underlined*) added to allow insertion into the unique *Eco*RI site in the lambda gt11 *lacZ* gene. Translation of the indicated reading frame yields a continuous sequence of 87 amino acids. *Bracketed numbers* below the amino acid sequence refer to tryptic fragments in Fig. 2A and the following *dotted lines* indicate the length of the corresponding identical amino acid sequence for each fragment of this sequence

Discussion

Although partial immunological cross-reactivity between phytase and pH 6.0-optimum acid phosphatase was observed, we were successful in using immunoscreening to detect at least four positive clones from the lambda gt11 library. These clones appear to be identical, based on restriction enzyme digest, and expressed an 87-amino-acid fragment from the DNA insert that the polyclonal antisera prepared from native phytase was able to identify. Although these clones do not contain the complete gene, this result was not totally unexpected due to the large size of the phytase protein at approximately 600 amino acids (Ullah 1988).

The initial concern was whether these clones contained a partial insert of the phytase gene or the pH 6.0-optimum acid phosphatase gene. Tryptic digest maps of both phytase and the pH 6.0-optimum acid phosphatase were not identical, suggesting that the conserved region(s) recognized by the antibody was not large. In particular, fragments 20, 30, 31, and 41 were sequenced, and the amino acid sequence obtained was present in the deduced amino acid sequence of the DNA insert. Working from the tryptic digest map of four sequenced amino acid regions, we were able to find the identical regions in the DNA sequence of the A-3 clone.

The recombinant phage lambda gt11 clone will make an excellent probe for isolating the rest of the gene from another library. We anticipate that the phytase gene itself will be large, based upon the approximately 600 amino acids present in the 85 kDa protein (Ullah 1988). At the present time, we do not have any information as to the presence of exons and introns within the gene, although other fungal genes such as glucoamylase (Innis et al. 1985) are comprised of several intervening regions.

In this report, we have been able to successfully use a polyclonal antiserum exhibiting cross-reactivity with another protein to isolate positive clones for the gene of interest. Using a combination of protein chemistry and DNA sequencing, we have been able to confirm that the recombinant clones are expressing a portion of the phytase gene. By providing a probe to clone this gene, the technique has advanced us towards our goal of reducing the phosphate load on the environment by developing an inexpensive source of fungal phytase. This work also shows the potential of these methods to clone other fungal genes coding for other useful proteins.

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