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Aquaculture 275 (2008) 70–75
Aquaculture
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A novel phytase gene *appA* from *Buttiauxella* sp. GC21 isolated from grass carp intestine

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Received 8 November 2007; received in revised form 17 January 2008; accepted 17 January 2008

Abstract

A novel phytase gene, *appA*, was isolated by degenerate PCR and genomic library screening from *Buttiauxella* sp. GC21, a bacterium isolated from grass carp intestine. The full-length gene consists of an open reading frame of 1341 bp and encodes 446 amino acids, including 33 amino acid residues of a putative signal peptide. Containing the active site of typical histidine acid phosphatases, the amino acid sequence shows maximum identity (71%) to PhyA from *Obesumbacterium proteus*. The gene encoding the mature phytase was expressed in *Escherichia coli* and the purified recombinant APPA had a specific activity for sodium phytate of 1180 U mg^{-1} of protein. The optimum temperature was 55 °C and the optimum pH was 4.5. The K_m value was 0.36 mM, with a V_{max} of 1.39 mmol min^{-1} mg^{-1}. This is the first report to detect and isolate a phytase from *Buttiauxella* sp.

Keywords: *Buttiauxella* sp.; Grass carp; Phytase; Phytate

1. Introduction

Phytate, or *myo*-inositol hexaphosphate (IP6), is the main storage form of phosphorus in plants (Reddy et al., 1989). Up to 80% of the total phosphorous content of plant proteins in fish feed may be present in the form of phytate (Jongbloed et al., 1997; Lott et al., 2000). Phytate can cause serious environmental pollution by phosphorus discharge since monogastric or agastric fish animals lack intestinal phytases and thus fail efficient digestion of phytate (Simon and Versteegh, 1990; Jackson et al., 1996). Furthermore, phytates form insoluble chelate complexes with nutritionally important metals and proteins, thereby decreasing their absorption and bioavailability (Harland and Morris, 1995; Vats and Banerjee, 2004).

Phytases (*myo*-inositol hexaphosphate phosphohydrolases, E.C.3.1.3.8) are the principle enzymes that specifically hydrolyze indigestible plant phytate into the lesser phosphorylated *myo*-inositol derivatives and inorganic phosphates (Mullaney and Ullah, 2003). Several bacterial phytase genes have been cloned from *Bacillus* sp. (Kim et al., 1998), *Escherichia coli* (Rodriquez et al., 1999; Golovan et al., 2000), *Klebsiella* sp. (Sajidan et al., 2004), *Obesumbacterium proteus* (Zinin et al., 2004), *Pseudomonas syringae* (Cho et al., 2005), *Yersinia intermedia* (Huang et al., 2006) and *Citrobacter* sp. (Luo et al., 2007).

The addition of phytases to monogastric animal feed has received considerable attention in recent years. Phytate has been reported to be susceptible to microbial and enzymatic degradation, however, only a few phytases have been identified in the aquatic environment to date (Suzumura and Kamatani, 1995). Due to the lack of basic research and inefficient processing technology, the utilization of phytase in fish feed is still in the initial stage compared with that of in poultry and swine feed (Baruah et al., 2005). Therefore, a novel

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doi:10.1016/j.aquaculture.2008.01.021

microbial phytase with a broad pH range, thermal stability and high specific activity could be an economic and efficient alternative to the phytases currently used in fish feed (Haefner et al., 2005).

In this study, we first isolated a phytase-producing strain of *Buttiauxella*, designed GC21, from the intestine of grass carp. A novel phytase gene was cloned and over-expressed in *E. coli*. The recombinant phytase was then purified and characterized.

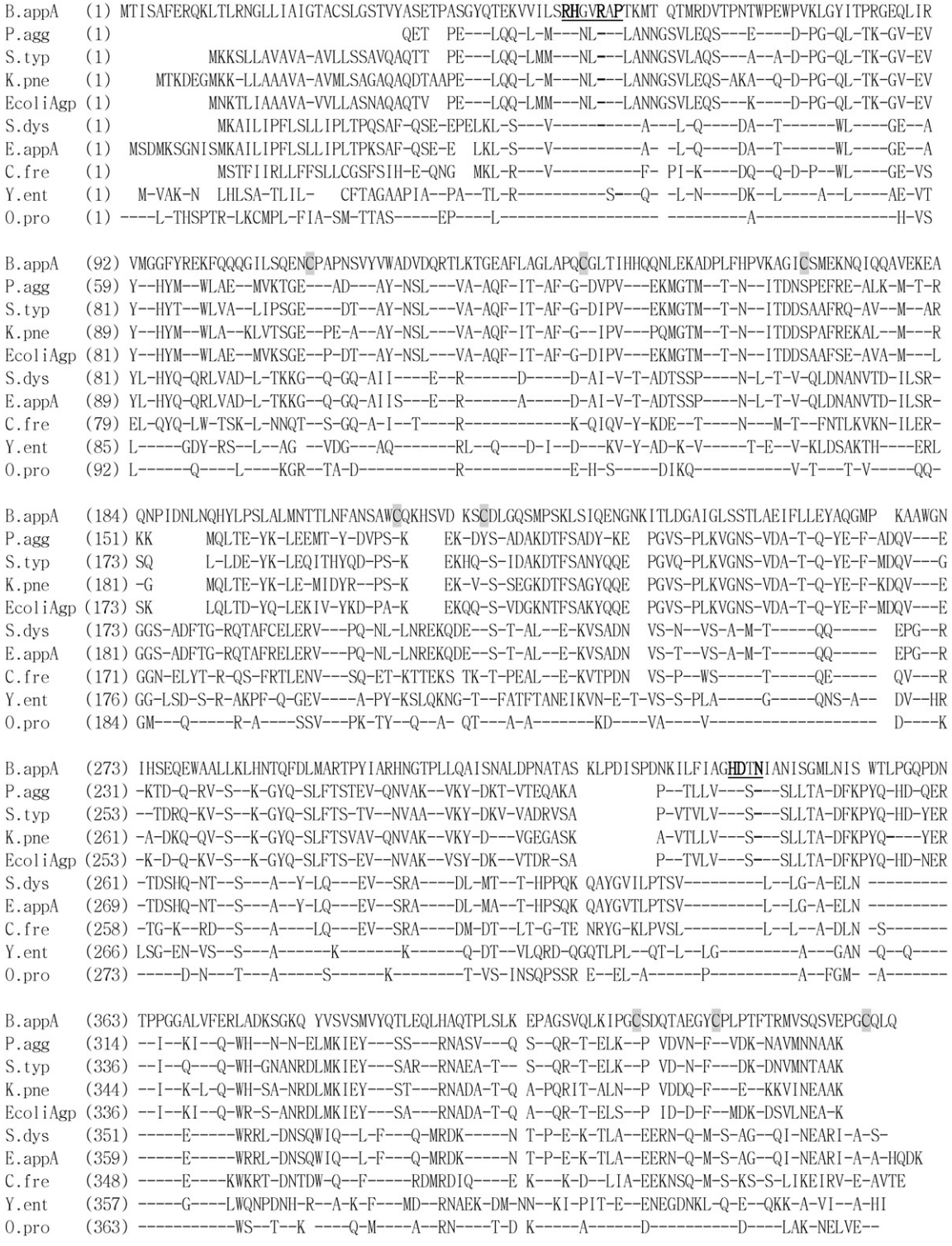

Fig. 1. Multiple alignment of *Buttiauxella* sp. GC21 phytase APPA homologs. The conserved histidine acid phosphatase family motifs are underlined; cysteine residues are shaded in gray. The abbreviations, source and GenBank accession nos. of proteins are: B. appA, *Buttiauxella* sp. GC21, EU159561; P.agg, *Pantoea agglomerans*, DQ435815; S.typ, *Salmonella typhimurium* LT2, AE008748; K.pne, *Klebsiella pneumoniae*, AY091638; EcoliAgp, *E. coli* O157:H7 EDL933, AE005174; S.dys, *Shigella dysenteriae* Sd197, CP000034; E.appA, *Escherichia coli* CFT073, AE014075; C.fre, *Citrobacter freundii*, AY390262; Y.ent, *Yersinia enterocolitica* subsp. enterocolitica 8081, AM286415; O.pro, *Obesumbacterium proteus*, AY378096.

2. Materials and methods

2.1. Strains, plasmids, and chemicals

E. coli JM109 and BL21 (DE3) cells were maintained in Luria–Bertani (LB) broth or agar medium at 37 °C for recombinant plasmid amplification and protein expression, respectively. The plasmids pUC19 (Takara, Dalian, China) and pGEM-T Easy vector (Promega, Madison, WI) were used for plasmid preparation and gene cloning, respectively. The pET-22b(+) vector (Novagen, San Diego, CA) was used for gene expression. All chemicals were of analytical grade.

2.2. Microorganism and culture conditions

Buttiauxella sp. GC21 was isolated from the intestine of grass carp in YPG medium (0.2% yeast extract, 0.1% peptone, 0.2% glucose, pH 7.2) and identified by using molecular methods (GenBank accession no. EU159562 for 16S rDNA sequence). For phytase production, *Buttiauxella* sp. GC21 was cultivated in 50 mL Erlenmeyer flasks containing 20 mL medium composed of 1% phytic acid calcium salt, 0.1% glucose, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$ and 0.03% $MnSO_4{\cdot}4H_2O$ at pH 7.2. The culture was incubated under shaking (220 rpm) conditions at 37 °C for 3 days.

2.3. Cloning of the phytase gene

The core region of the phytase gene, *appA*, was obtained using PCR with degenerate primers F1 (5'-GTK STK AWW KTG AGY CGC CA-3') and R1 (5'-TWK GCM AKR TTR GTA TCA TG-3') designed on the basis of two conserved amino acid sequences RHGXRXP and HDTN among histidine acid phosphatase (HAP) family from *Enterobacteriaceae* (Huang et al., 2006). The amplified fragment (850–900 bp) was purified and ligated into pGEM-T Easy vector for sequencing and BLAST analysis.

A genomic library was constructed as described by Yang et al. (2007) with slight modifications. The chromosomal DNA of *Buttiauxella* sp. GC21 was digested with *Sau3*AI and electrophoresed on a 0.8% agarose gel. The 5–10 kb fragments were purified from the gel, ligated into the *Bam*HI site of pUC19, and then transformed into *E. coli* JM109 cells to construct the genomic DNA library. A colony PCR method was used for the target gene isolation using two specific synthetic primers: BF (5'-CTC AGT TTA CGT CTG GGC AG-3') and BR (5'-GTT ATG CAG TTT CAG CAA CGC C-3'). A positive clone was obtained, and the recombinant plasmid was isolated and sequenced. The nucleotide sequence for the phytase gene from *Buttiauxella* sp. GC21 was deposited in GenBank under the accession no. EU159561.

2.4. Sequence and phylogenetic analysis

The sequence assembly was performed using the Vector NTI Suite 7.0 software, and the nucleotide sequence was analyzed using the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The presence of a signal peptide in the deduced amino acid sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The DNA and protein sequence alignments were carried out using the blastn and blastp programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments of protein sequences were performed using the CLUSTALW program (http://www.ebi.ac.uk/clustalW/). A phylogenetic tree was constructed using the Neighbor–Joining (NJ) algorithm in MEGA version 3.1, with its reliability assessed by 1000 bootstrap repetitions.

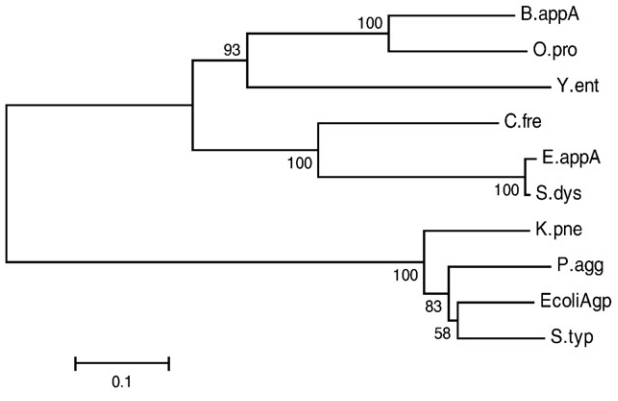

Fig. 2. Phylogenetic tree of the *Buttiauxella* sp. GC21 phytase APPA homologs. Bootstrap values (%) from analysis of 1000 bootstrap replicates are given at the respective nodes. See Fig. 1 for abbreviations.

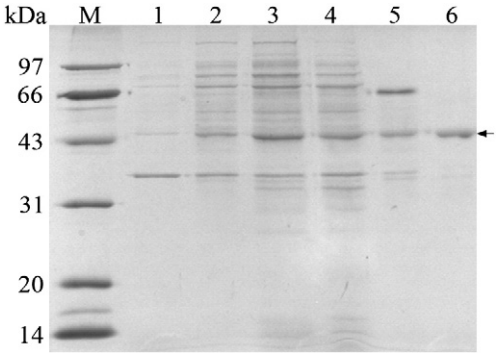

Fig. 3. SDS-PAGE analysis of recombinant APPA expressed in the culture supernatant of *E. coli* BL21 (DE3) cells. Lane M, standard protein molecular weight markers (in kDa); lane 1, BL21 harboring empty pET-22b(+) vector induced with IPTG; lane 2, transformant harboring pET-BappA uninduced with IPTG; lane 3, transformant harboring pET-BappA induced with 0.6 mM IPTG at 37 °C for 4 h; lane 4, crude enzyme preparation; lane 5, recombinant APPA after purification on a HiTrap Q Sepharose XL column; lane 6, recombinant APPA after purification on a Sephacryl S-200 HR column. The arrow indicates the position of APPA.

2.5. Expression of appA in E. coli

PCR amplification of DNA containing the core region of *appA* without the signal peptide was performed using primers BappAF (forward, 5′-TT *CCA TGG* ATA GCG AAA CTC CCG CTT CAG GTT ATC-3′) and BappAR (reverse, 5′-AA *GAA TTC* TTA CTG TAG CTG GCA ACC CGG TTC-3′) (the *Nco*I and *Eco*RI restriction sites in BappAF and BappAR are italic, respectively). The PCR product was then gel-purified, digested with *Nco*I and *Eco*RI, and cloned into the corresponding sites of the pET-22b(+) vector. The recombinant plasmid, pET-BappA, was transformed into *E. coli* BL21 (DE3) competent cells.

A positive transformant harboring pET-BappA was picked from a single colony and grown overnight at 37 °C in LB medium supplemented with ampicillin (100 μg mL^{-1}). The culture was then inoculated into fresh LB medium (1:100 dilution) containing ampicillin and grown aerobically at 37 °C, with shaking to an optical density of OD_{600}=0.6–0.8. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was then added at a final concentration of 1.0 mM. The cultures were grown for an additional 4 h at 37 °C or 6 h at 30 °C, and the expressed APPA was analyzed by SDS-PAGE.

2.6. Purification of recombinant APPA

The cell-free supernatant was harvested by centrifugation, and precipitated by 60–80% ammonium sulfate saturation followed by recentrifugation. The precipitate was then suspended in 20 mM Tris–HCl (pH 8.0), dialyzed against the same buffer, and concentrated using polyethylene glycol 8000. The clear supernatant was loaded onto a HiTrap Q Sepharose XL column (GE Healthcare, Uppsala, Sweden) equilibrated with the same buffer. The fractions exhibiting phytase activity were pooled, concentrated, and stored at 4 °C before use. The concentrated supernatant was loaded onto a Sephacryl S-200 column that had been equilibrated with 20 mM Tris–HCl (pH 8.0) for two column volumes at a rate of 0.5 mL min^{-1} until the baseline was stable. Protein was eluted with one column volume of the same buffer at a flow rate of 1.0 mL min^{-1} and fractions

with enzyme activity were collected based on the protein concentration as detected at OD_{280}. The protein concentration was assayed by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

2.7. Enzyme activity and properties of recombinant APPA

Phytase activity was determined by the ferrous sulfate-molybdenum blue method according to Holman (1943). One unit of phytase activity was defined as the amount of enzyme required to liberate 1 μmol phosphate per min at 37 °C.

For the pH profile, enzyme activity was assayed using the following buffers: glycine-HCl, pH 2.0–3.5; sodium acetate-acetic acid, pH 3.5–6.0; Tris–HCl, pH 6.0–8.5; and glycine–NaOH, pH 8.5–11.0. The buffers used for diluting the recombinant enzyme contained 0.05% (w/v) bovine serum albumin and 0.05% (w/v) Triton X-100. The effect of pH on enzyme stability was estimated in the same buffer system from pH 2.0 to 10.0. The optimum temperature was determined at the optimum pH at temperatures ranging from 30 to 80 °C. The temperature stability was estimated by incubation of the enzyme in 50 mM buffers at 50, 60, and 70 °C for 2, 5, 10, 20, 30, 40, 50, and 60 min. The effects of different metal ions and reagents (1 mM each) on the phytase activity were determined.

The K_m and V_{max} values for phytase were determined by Lineweaver–Burk analysis. The enzyme activity was measured at 37 °C in 0.25 M sodium acetate (pH 4.5) containing 0.125 to 4.0 mM sodium phytate as substrate.

3. Results

3.1. Cloning of the phytase gene appA

The core region of *appA* was amplified by PCR using the degenerate primers, and the product was cloned into pGEM-T Easy vector. Sequencing showed that the core region comprised 899 bp. Based on the partially identified sequence, BF and BR primers were synthesized and used for screening of the *Buttiauxella* sp. GC21 genomic library. Approximately 6000 recombinants were screened, and only one clone, pUCB-2008, was found to contain the gene *appA*. The insert of the plasmid DNA from pUCB-2008 was approximately 3.6 kb. After sequencing, one complete open reading frame of 1341 bp was found.

3.2. Sequence and phylogenetic analysis

Sequence analysis reveals that *appA* consists of a signal peptide of 33 amino acid residues and a mature phytase domain of 413 residues with a calculated molecular mass of 45.3 kDa. Nine APPA homologs were found in GenBank using BLAST and aligned (Fig. 1). A phylogenetic tree was constructed based on the alignment (Fig. 2). All proteins were isolated from *Enterobacteriaceae*. The closest homologs to APPA were PhyA from *O. proteus* (71% identity) and a probable histidine acid phosphatase from *Y. enterocolitica* (51% identity). The BLAST results identified APPA as a member of the histidine acid phosphatase (HAP) family with the conserved active sites of RHGVRXP and HD (Fig. 1, underlined). Eight cysteine residues that theoretically form four disulfide bonds were also found in APPA (Fig. 1, highlighted).

3.3. Overexpression of appA in E. coli

The recombinant plasmid pET-BappA was constructed and *E. coli* transformants containing the plasmid were screened for phytase activity. The cell lysate and medium supernatant showed a phytase activity of 41.6 U mL^{-1} and 14.2 U mL^{-1}, respectively. The uninduced transformant and the transformant harboring the empty pET-22b(+) vector showed no activity. The recombinant enzyme was then visualized by SDS-PAGE and Coomassie staining, and the molecular mass was about 45.0 kDa, which is close to the theoretical value (Fig. 3).

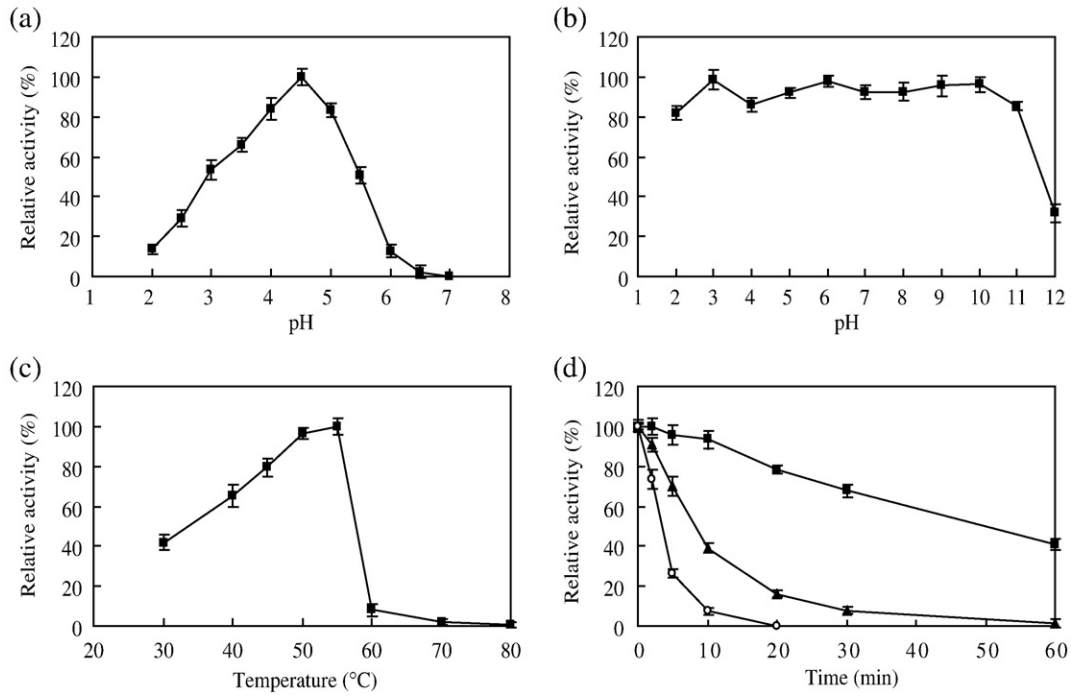

Fig. 4. Characterization of recombinant APPA. (a) Effect of pH on phytase activity. The phytase activity was measured at 37 °C in pH 2.0–7.0. (b) pH stability of recombinant APPA. After incubating recombinant APPA at 37 °C for 1 h in buffers with pHs ranging from 2.0–12.0, the residual activity was measured under standard assay conditions. (c) Effects of temperature on phytase activity. The activity was measured at the indicated temperatures and at pH 4.5. (d) Thermostability of recombinant APPA. The enzyme was pre-incubated at 50 °C (■), 60 °C (▲), or 70 °C (○) for different lengths of time, and aliquots were removed at specific time points to measure the residual activity under standard assay conditions.

Table 1
Effects of metal ions and reagents on the enzyme activity of purified recombinant APPA

Reagent[a]	None	Fe^{3+}	Li^{+}	Co^{2+}	Ni^{2+}	Mn^{2+}	Cr^{3+}	Hg^{2+}
Relativity phytase activity (%)	100.0	3.0	107.8	93.9	82.9	100.6	80.1	2.8
Reagent[a]	Na^{+}	K^{+}	Ca^{2+}	Cu^{2+}	Mg^{2+}	Zn^{2+}	EDTA	SDS
Relativity phytase activity (%)	96.1	96.5	101.1	9.6	100.0	15.0	98.0	0

[a] The concentration of each reagent was 1 mM in the assay buffer.

3.4. Purification of recombinant APPA

The recombinant phytase was purified to electrophoretic homogeneity from the culture supernatant by ammonium sulfate precipitation, anion exchange chromatography, and gel filtration chromatography. As a result, the specific activity of the purified recombinant APPA was 1180 U mg^{-1} after 7.8-fold purification, with a final activity yield of 12.7%. The purified enzyme yielded a single band with a molecular mass of 45.0 kDa, as determined by SDS-PAGE (Fig. 3).

3.5. Properties of the recombinant APPA

The purified recombinant APPA was active at acidic pH values (2.0–6.0), and the optimum pH was 4.5 (Fig. 4a). The enzyme activity was stable over a wide range of pH, retaining over 80% of the total activity after incubation at pH 2.0 to 11.0 (Fig. 4b). The maximal activity was observed at 55 °C (Fig. 4c). The phytase remained quite stable at 50 °C after incubation at pH 4.5 for 30 min, but lost activity very rapidly when the temperature was further increased (Fig. 4d).

The effect of various metal ions and reagents on enzyme activity were also determined (Table 1). Residual activities of the enzyme were not affected by most of the metal ions and reagents under test (1 mM); however, the activity was partially inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+} and Fe^{3+}, and completely inhibited by SDS.

The kinetic parameters for the hydrolysis of phytate have a K_m of 0.36 mM and a V_{max} of 1.39 mmol min^{-1} mg^{-1} as determined from a Lineweaver–Burke plot. The specific activity of the phytase was 1180 U mg^{-1} of protein.

4. Discussion

The easily decomposable nature of phytate makes it a potential producer of phosphate in the aquatic ecosystem and may cause serious environmental problems (Haefner et al., 2005). Therefore, application of phytases to fish feed increases phytate digestion, and therefore solves the environmental problems (Cao et al., 2007). In this study, we isolated some phytate-degrading bacteria from the intestine of grass carp, which only feed on *Lolium perenne*. Most of the isolates may produce neutral or alkaline phytases, such as *Bacillus* sp. (Kim et al., 1998), *Shewanella* sp. (Cheng and Kim, 2006), and *Pseudomonas* sp. (Dharmsthiti et al., 2005), since their phytase activity can be detected at pH 7.5 (data not shown). Two isolates, *Buttiauxella* sp. GC21 and *Hafnia* sp. GC36, produced intracellular acidic phosphatases, as evidenced by the fact that their enzymatic activity could only be detected at acidic pH. From the perspective of nutrition, phytases from fish intestinal microbes may not only increase the bioactivity of some minerals by breaking down the bonds between minerals and phytate, but also become potential candidates for the production of special isomers of different lower phosphate esters of *myo*-inositol, which is classified as a vitamin-like compound and is necessary to some fish species, including red sea bream, common carp, and Atlantic salmon, at a certain stage of growth (Shiau and Su, 2004). Therefore, these phytases might be important for diet formulation, development status of the digestive system, and the growth performance of some fish.

The core regions of acidic phytase from *Hafnia* sp. GC36 and *Buttiauxella* sp. GC21 showed 98% and 73% identity to PhyA from *O. proteus*, respectively, indicating that a novel phytase gene has been cloned from *Buttiauxella* sp. that belongs to the same subclass of the HAP family as *O. proteus* (Zinin et al., 2004). Based on the position of the active site and the cysteine residues, we suggest that these proteins may have similar three-dimensional structures as well as similar enzyme mechanisms (Lim et al., 2000).

The recombinant APPA was successfully expressed and secreted in *E. coli* in an inducible system. The recombinant APPA was optimally active at pH 4.5 and retained more than 80% of its activity after incubation at a wide range of pH (2.0–11.0). This enzymatic characteristic allows for a stable phytase under the different pH conditions encountered in feed processing, storage and the fish digestive tract. The APPA exhibited a temperature optimum of 55 °C. This high optimum temperature and its thermostability preclude the full activity of phytase at relatively lower temperature of the stomach and intestines of fish (Lei and Porres, 2003). The specific activity of the purified APPA (1180 U mg^{-1}) is almost 4-fold that from *O. proteus* (310 U mg^{-1}), suggesting its potential as an alternative to the present feed additives. Moreover, the enzymatic properties of APPA may be improved by protein engineering (Haefner et al., 2005).

In conclusion, a novel phytase gene was isolated from *Buttiauxella* sp. GC21 that was isolated from the intestine of grass carp. The phytase gene could be successfully expressed in *E. coli* with high expression level. The successful purification and characterization of the enzyme given here provides a basis to further large-scale production and suggests possible application in fish feed.

Acknowledgements

This work was funded by the National High Technology Research and Development Program of China (863 Program, grant no. 2006AA020101) and the National Basic Research Program of China (973 Program, grant no. 2004CB719606).

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