Appl Microbiol Biotechnol (2006) 70: 60–64
DOI 10.1007/s00253-005-0024-8

T. Herter · O. V. Berezina · N. V. Zinin · G. A. Velikodvorskaya · R. Greiner · R. Borriss

Glucose-1-phosphatase (AgpE) from *Enterobacter cloacae* displays enhanced phytase activity

Received: 30 March 2005 / Revised: 18 May 2005 / Accepted: 20 May 2005 / Published online: 28 September 2005

Abstract Using a screening procedure developed for detection of phytate hydrolysing enzymes, the gene *agpE* encoding glucose-1-phosphatase was cloned from an *Enterobacter cloacae* VKPM B2254 plasmid library. Sequence analysis revealed 78% identity on nucleotide and 79% identity on peptide level to *Escherichia coli* glucose-1-phosphatase characterising the respective gene product as a representative of acid histidine phosphatases harbouring the RH(G/N)RXRP motif. The purified recombinant protein displayed maximum specific activity of 196 U mg^{-1} protein against glucose-1-phosphate but was also active against other sugar phosphates and *p*-nitrophenyl phosphate. High-performance ion chromatography of hydrolysis products revealed that AgpE can act as a 3-phytase but is only able to cleave off the third phosphate group from the *myo*-inositol sugar ring. Based on sequence comparison and catalytic behaviour against phytate, we propose to classify bacterial acid histidine phosphatases/phytases in the three following subclasses: (1) AppA-related phytases, (2) PhyK-related phytases and (3) Agp-related phytases. A distinguished activity of 32 U mg^{-1} of protein towards *myo*-inositol-hexa-phosphate, which is two times higher than that of *E. coli* Agp, suggests that possibly functional differences in terms of phytase activity between Agp- and AppA-like acid histidine phosphatases are fluent.

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00253-005-0024-8 and accessible for authorised users.

T. Herter · R. Borriss (✉)
Institute of Biology, Bacterial Genetics, Humboldt University, Berlin, Germany
e-mail: rainer.borriss@rz.hu-berlin.de

O. V. Berezina · G. A. Velikodvorskaya
Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russian Federation

N. V. Zinin
State Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russian Federation

R. Greiner
Federal Research Centre of Nutrition and Food, Centre for Molecular Biology, Karlsruhe, Germany

Introduction

Phytase is a general term describing an enzyme that initiates the hydrolysis of phosphomonoester bonds from phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate, $InsP_6$), thereby liberating inorganic orthophosphate (Mullaney and Ullah 2003). Phytases are used as an animal feed additive to improve phosphate bioavailability and to reduce the loss of phosphate and divalent cations from phytate, which represents the principal storage form of phosphorous in plant seeds. According to their pH optimum, acid and alkaline phytases can be distinguished (Oh et al. 2004). Acid phytases evolved from acid histidine acid phosphatases (HAPs, EC 3.1.3.8), which display general phosphomonoesterase activity directed against a broad spectrum of phosphate-containing substrates including phenyl- and naphthyl-*ortho*-phosphates. A minority of HAPs are able to hydrolyse phytate with varying degrees of efficiency. The phytase enzyme with the highest specific activity presently known is the pH 2.5 acid phosphatase AppA from *Escherichia coli* (Golovan et al. 2000). In contrast, *E. coli* acid glucose-1-phosphatase (Agp, EC 3.1.3.10), which, despite its apparent sequence similarity to AppA, primarily cleaves small monosaccharide phosphates such as glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate, but it also exhibits phytase activity (Cottrill et al. 2002). Occurrence and characterisation of Agp in bacteria other than *E. coli* has not been reported so far.

Previous experiments revealed that representatives of the genus *Enterobacter* can hydrolyse phytate (Sajidan 2002). Here, we have cloned the gene *agpE*, which encodes an enzyme that hydrolyses glucose-1-phosphate and phytate, from an *Enterobacter cloacae* VKPM B2254 plasmid library. In addition, a second *agpE* gene was cloned from strain ASR5 sharing high 16S rDNA sequence similarity

with that of *E. cloacae*. The deduced amino acid sequence of *agpE* was nearly 80% identical with that of the *E. coli agp* gene, and the biochemical properties of the expressed recombinant gene product were found to be similar to those reported for *E. coli* Agp except 3-phytase activity that exceeded that of its *E. coli* counterpart two times.

Materials and methods

E. cloacae VKPM B-2254 was obtained from S.P. Sineoky, Moscow, and is deposited in the strain collection of the State Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia. *Enterobacter* sp. ASR5 strain was isolated and taxonomically characterised by Sajidan (2002). According to its 16S rDNA sequence strain, ASR5 was found highly related to *E. cloacae* and was deposited in the DSMZ culture collection as *Enterobacter* sp. DSM 17269. *E. cloacae* DNA was partly digested with *Sau*3A and ligated into *Bam*HI-linearised pUCI9 and cloned into *E. coli* XL1-Blue. The resulting *E. cloacae* genome library contained about 10,000 clones with insert sizes ranging from 5 to 8 kb. Transformants were screened for phytase activity on plates as previously described (Zinin et al. 2004). Plasmid DNA of two clones expressing phytase activity was isolated and found to harbour overlapping inserts of 6.1 and 5.3 kb. After appropriate subcloning, the insert DNA containing the glucose-1-phosphatase gene was sequenced in both directions using standard primers [pUC/M13 (forward), pUC/M13 (reverse)] and specific primers (pOB1, forward 1,261–1,280: 5′-acgctggagctgaaaggctg-3′; pOB2, reverse 670–651: 5′-tcatctgctccagcagctta-3′; and pOB3, reverse 261–242: 5′-gacggctcatgattaaaact-3′). DNA sequences were determined with an automatic sequencing system (ALF, Pharmacia). Sequence analysis was performed with the BLAST, ClustalW (Thompson et al. 1994) and phylogenetic analysis using parsimony (PAUP; Swofford 2002) programme packages. To clone the *agpE* gene in the expression pET 22b(+) vector, the gene was amplified using the primers OB_for1, forward: 5′-agtgaggaattacatatgagaaaagcac-3′, and OB_rev, reverse: 5′-tgaattcgccgcgttattcatcac-3′. The obtained DNA fragment was digested with *Nde*I and *Eco*RI, and inserted between the *Nde*I and *Eco*RI sites of the vector; ligation products were transferred into *E. coli* C41(DE3) (Miroux and Walker 1996) to test for enzyme activity. C41(DE3) transformants were cultured at 37°C in TBY containing ampicillin (50 μg ml^{-1}). At OD_{600}=0.6–0.8, phosphatase expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration 1 mM), and the cultures were further incubated at 37°C for 5 h. After centrifugation, glucose-1-phosphatase was purified from the supernatant by affinity chromatography with Ni-NT agarose (Electronic Supplementary Material, Fig. S1). Phytase and phosphatase activities were assayed at 37°C in 0.1 M sodium acetate (pH 5.0) as previously described (Sajidan et al. 2004). One unit of activity was defined as the amount of enzyme that liberates 1 μmol phosphate in 1 min at 37°C. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. Specific enzyme activities were defined in units per milligram protein and determined as previously described (Sajidan et al. 2004). The molecular mass of native AgpE was determined by FPLC-column chromatography (column TOSOHAAS TSK gel G2000 SWXL, 7.8×300 mm, particle size 5 μm) in 100 mM Tris–HCl, pH 7.5, and in 100 mM sodium acetate buffer, pH 6.0, supplemented with 150 mM sodium chloride. Products of phytate hydrolysis were determined as previously described (Sajidan et al. 2004). The coding sequence of the *agpE* gene from *E. cloacae* VPKM B2254 has been deposited in the EMBL nucleotide sequence data bank under accession number AJ783768.

Results and discussion

The *agpE* gene was isolated from a plasmid library prepared from genomic DNA from *E. cloacae* VPKM B2254, as described in Materials and methods. Sequence analysis revealed that an open reading frame with 78% nucleotide identity and 79% amino acid identity to *E. coli* periplasmic glucose-1-phosphatase (Agp) was present in both cloned DNA fragments. The *agpE* gene was flanked by two open reading frames, orf1 and orf2. The deduced amino acid sequences of orf1 and orf2 displayed 81% identity to the Trp-repressor binding protein from *Salmonella typhimurium* LT2 and 70% identity with a transcriptional regulatory protein from *Bradyrhizobium japonicum*, respectively (Electronic Supplementary Material, Fig. S2). The *agpE* gene product is a 413-amino acid protein with notable homology to HAPs. The 391-amino acid sequence of the putative mature protein corresponds to a molecular mass of 44 kDa, and it contains a variation of the active site signature motif RH*N*XRXP instead of RH*G*XRXP, which is common in HAPs and phytases (Mitchell et al. 1997).

Amino acid sequence alignment with available bacterial HAP/phytase sequences was performed to generate a phylogenetic tree, which was used as a starting point for maximum likelihood and maximum parsimony heuristic searches with bootstrap support. The topology of the resulting phylogenetic tree, which was very similar to that of the tree obtained by the neighbour joining method, is supported by the results of bootstrap repetition tests (Fig. 1). *E. cloacae* AgpE and *E. coli* Agp, together with the putative acid *Providencia rettgeri* glucose-1-phosphatase and the enterobacterial *S. flexeri* and *S. typhimurium* homologs, form a separate Agp branch distinguished by the unique RHNXRXP active site motif (Electronic Supplementary Material, Fig. S3). Other bacterial HAPs/phytases previously grouped as PhyC phytases (Oh et al. 2004) can be subclassified as AppA-related phytases, along with a more heterogenous group consisting of *Klebsiella* spp. and *Pseudomonas syringae* phytases, termed PhyK. AgpE shares about 30% identical residues with *E. coli* AppA and *Klebsiella* PhyK. *E. cloacae* AgpE and *E. coli* Agp, together with a few open reading frames identified in the completed genomes of several enterobacteria, constitute a

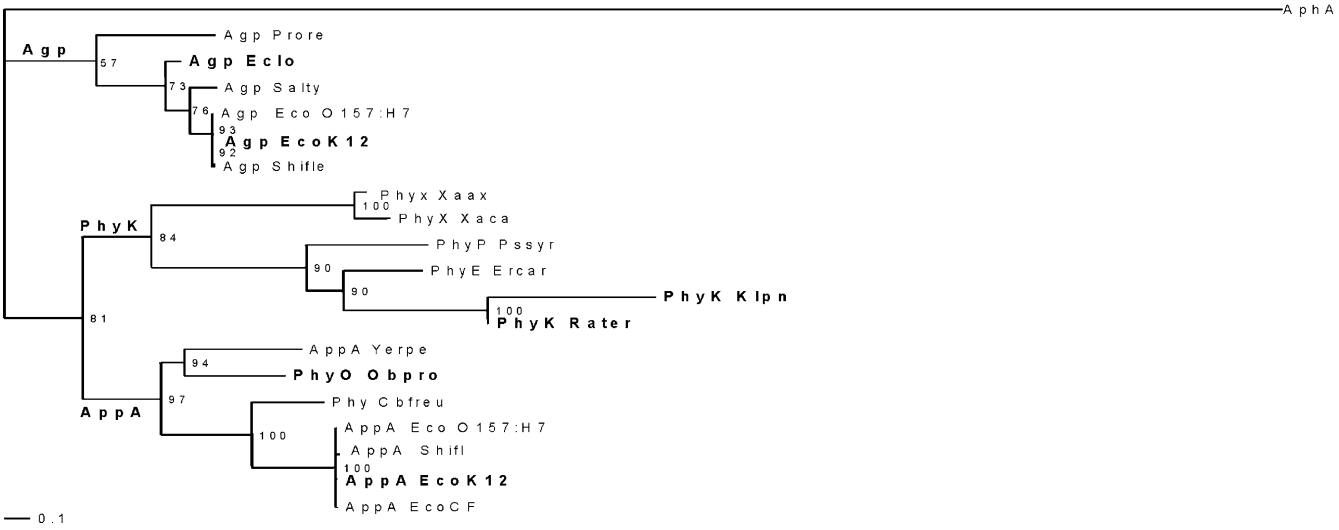

Fig. 1 Phylogenetic tree constructed by random stepwise parsimony using the PAUP programme package of homologs of the *Enterobacter cloacae* acid glucose-1-phosphatase (Agp). The bar represents one substitution per ten amino acids. Bootstrap values (%) from analysis of 1,000 bootstrap replicates are given at the respective nodes. Homologs with experimentally verified phytase activity are in bold letters. Histidine acid phosphatase (HAP) from *Klebsiella pneumoniae* ASR1-AphA Klpn (gi:18092533) was used as out-group. The abbreviations, source, gi accession numbers for experimental data, if available, of proteins are Agp Eclo (gi:50284400)—G1Pase *E. cloacae*; Agp EcoK12 (gi:145218)—G1Pase *Escherichia coli* K12; Agp Eco O157:H7 (gi:13360618)—G1Pase *E. coli* O157:H7; Agp Shifle (gi:30062539)—putative G1Pase *Shigella flexneri* genome; Agp Salty (gi:16764475)—putative G1Pase *Salmonella typhimurium* genome; Agp Prore (gi:45772)—putative G1Pase *Providencia rettgeri*; PhyX Xaax (gi:21244849)—phytase *Xanthomonas axonopodis*; PhyX Xaca (gi:21230234)—phytase *Xanthomonas campestris*; Phy Pssyr (gi:26280977)—putative phytase *Pseudomonas syringae* MOK1; PhyE Ercar (gi:50123118)—putative phytase *Erwinia carotovora*; PhyK Rater (gi:32451230)—phytase *Raoultella terrigena*; PhyK Klpn (gi:29465764)—phytase *K. pneumoniae*; AppA Yerpe (gi:45441589)—probable HAP *Yersinia pestis*; PhyO Obpro (gi:37266291)—phytase *Obesumbacterium proteus*; Phy Cbfreu (gi:40748277)—phytase *Citrobacter freundii*; AppA EcoK12 (gi:145285)—phosphoanhydride phosphohydrolase *E. coli* K12; AppA Eco O157:H7 (gi:25350609)—phosphoanhydride phosphohydrolase *E. coli* O157:H7; AppA Shifl (gi:24112393)—probable phosphoanhydride phosphohydrolase *S. flexneri*; AppA EcoCF (gi:26247006)—probable phosphoanhydride phosphohydrolase *E. coli* CFT073

separate branch clearly separated from the "true" phytases belonging to the AppA or PhyK groups (Fig. 1).

Using sequence-specific primers derived from the *agpE* sequence, we amplified another *agp* gene from the soil bacterium *Enterobacter* sp. strain ASR5 (DSM 17269), which was isolated from the rhizosphere of an Indonesian rice field, demonstrating that the gene is well conserved among different members of the genus *Enterobacter* and that it might be responsible for the phytase activity previously observed in this strain (Sajidan et al. 2004).

The apparent molecular mass of AgpE as determined by native gel filtration chromatography in 100 mM Tris–HCl buffer, pH 7.5, 150 mM NaCl, indicates that the enzyme has a dimeric form (Electronic Supplementary Material, Fig. S4b,d). Protein fractions corresponding to about 82.5 kDa cross-reacted with His-tag-specific antibodies and displayed glucose-1-phosphatase activity (Electronic Supplementary Material, Fig. S5). *E. coli* Agp was also reported to occur in a homodimeric state (Pradel and Bouquet 1988). Notably, pH conditions seem to affect the assembly of AgpE monomers. Complexes with a higher molecular mass of 125 kDa indicative of a homotrimeric AgpE were detected by native gel chromatography performed in sodium acetate buffer, pH 6 (Electronic Supplementary Material, Fig. S4a,c).

Besides glucose-1-phosphatase activity, purified AgpE was active towards other monosaccharide sugar phosphates: glucose-6-phosphate, fructose-1-phosphate and fructose-6-phosphate (Table 1). Activity towards phytate and the general phosphatase substrate *p*-nitrophenyl phos-

Table 1 Comparison of kinetic parameters of the *Enterobacter cloacae* AgpE with *E. coli* Agp with various substrates

Substrate	K_m (mM)		Specific activity (U mg^{-1})		k_{cat} (s^{-1})		k_{cat}/K_m (s^{-1} mM^{-1})	
	AgpE	*E. coli* Agp	AgpE	*E. coli* Agp	AgpE	*E. coli* Agp	AgpE	*E. coli* Agp
$InsP_6$	0.51	0.54	32	16	24	12	47	22
Glucose-1-phosphate	0.31	0.39	196	160	145	117	464	300
Glucose-6-phosphate	0.47	1.6	146	110	109	81	230	51
Fructose-1-phosphate	0.62	2.2	159	130	111	95	179	43
Fructose-6-phosphate	0.66	4.3	142	79	105	58	160	13
p-Nitrophenyl phosphate	13	13	40	51	30	37	2.4	2.8

Data for *E. coli* Agp were extracted from Cottrill et al. 2002

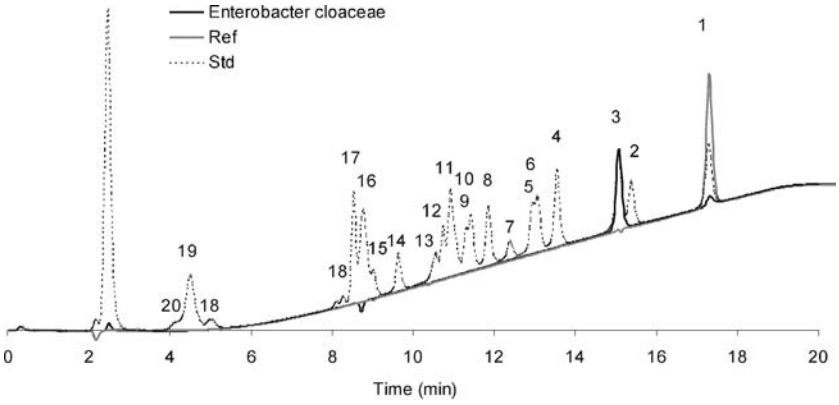

Fig. 2 Hydrolysis of *myo*-inositol hexakisphosphate by the purified recombinant AgpE. Enzyme and substrate were incubated at pH 5.0 and reaction products were analysed by high-performance ion chromatography (HPIC) (see Materials and methods). Action of purified recombinant glucose-1-phosphate on sodium phytate is indicated as a black line. Peaks of reference *myo*-inositol phosphates are shown in dotted lines: (1) Ins(1,2,3,4,5,6)P_6; (2) Ins(1,3,4,5,6)P_5; (3) D/L-Ins(1,2,4,5,6)P_5; (4) D/L-Ins(1,2,3,4,5)P_5; (5) Ins(1,2,3,4,6)P_5; (6) D/L-Ins(1,4,5,6)P_4; (7) Ins(2,4,5,6)P_4; (8) D/L-Ins(1,2,5,6)P_4; (9) D/L-Ins(1,3,4,5)P_4; (10) D/L-Ins(1,2,4,5)P_4; (11) D/L-Ins(1,2,3,4)P_4; (12) D/L-Ins(1,2,4,6)P_4; (13) Ins(1,2,3,5)P_4; (14) D/L-Ins(1,5,6)P_3; (15) D/L-Ins(1,4,5)P_3; (16) D/L-Ins(1,2,6)P_3, Ins(1,2,3)P_3; (17) D/L-Ins(1,3,4)P_3; (18) D/L-Ins(1,2,4)P_3, D/L-Ins(2,4,5)P_3, (19) D/L-Ins(2,4)P_2; (20) D/L-Ins(1,2)P_2, Ins(2,5)P_2, D/L-Ins(4,5)P_2; (21) D/L-Ins(1,4)P_2, D/L-Ins(1,6)P_2

phate was also detected. Compared with the preferred substrate glucose-1-phosphate, the phytase activity of AgpE was found to be six- to sevenfold lower, corresponding to about 2% to 25% of the activities reported for true HAP phytases such as *E. coli* AppA (Zinin et al. 2004) and *Klebsiella* sp. ASR1 PhyK (Sajidan et al. 2004). On the other hand, the specific phytase activity of 32 U mg^{-1} lies in the same range or slightly higher than that reported for alkaline *Bacillus* phytases (Tye et al. 2002) and exceeds that of its *E. coli* counterpart two times. Interestingly, AgpE displayed a K_m value for phytate, which is in the same range as that reported for AppA (Table 1). In order to examine the action of the phytate-degrading enzyme on phytate, hydrolysis products were separated by isomer-specific high-performance ion chromatography (HPIC). A marked decrease in the $InsP_6$ (phytate) content of the reaction mixture with a concomitant increase in *myo*-inositol pentakisphophate content was observed during incubation. In contrast, *myo*-inositol phosphates with fewer than five phosphate residues were not detected throughout the incubation period (Fig. 2). These data demonstrate that the *E. cloacae* phytate-degrading enzyme dephosphorylates phytate quantitatively to a single *myo*-inositol pen takis phosphate isomer . On the contrary, the related *E. coli* 6-phytase AppA can dephosphorylate $InsP_6$ in a stepwise manner to yield the Ins-2-monophosphate (Greiner et al. 2000). Also, a 3-phytase from *Klebsiella* ASR1, a representative of the PhyK group, was reported to gradually dephosphorylate phytate to Ins(2)P (Sajidan et al. 2004). The reported 3-dimensional structure of the AppA/phytate complex demonstrates that phytate initially does not fully occupy the flexible AppA binding pocket, but after binding the pocket, perfectly conforms to the substrate allowing its successive degradation (Lim et al. 2000). In contrast, the comparatively stiff and small substrate binding cleft of Agp allows only for the orientation for phytate in which the 3-phosphate is docked at the catalytic site of the enzyme. Leu-24 and Glu-196 in *E. coli* Agp have been proposed to act as "gating residues" by narrowing access to the substrate (Lee et al. 2003). Both residues are conserved in all Agp-related sequences including AgpE but are absent from the PhyK- and AppA-related homologs (Supplementary Material, Fig. S2). AgpE clearly belongs to the group of HAPs with main activity directed towards glucose-1-phosphate, but its phytase activity is striking higher than that reported for *E. coli* Agp, demonstrating that there is considerable variability in ratio of both enzymatic activities possibly caused by slight differences in the environment of the active site area. Investigation of further Agps from other bacterial sources might support this hypothesis.

Acknowledgements We thank Dr. Sineoky, Moscow, for providing the *Enterobacter cloacae* strain used in this study and Mrs. Christiane Müller, Berlin, for excellent technical assistance. We are much obliged to Kathleen Smith, Paris and the unknown reviewers for many valuable suggestions improving the manuscript. Dr. Andreas Weihe, Berlin, is especially thanked for his support in using the PAUP package for construction of the evolutionary tree. We are very grateful to BMBF for the continuous financial support given in the framework of the exchange project RUS 01/235.

References

Bradford MM (1976) A rapid sensitive method fort the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Cottrill MA, Golovan SP, Philipps JP, Forsberg CW (2002) Inositol phosphatase activity of the *Escherichia coliagp*-encoded acid glucose-1-phosphatase. Can J Microbiol 48:801–809

Golovan S, Wang G, Zhang J, Forsberg CW (2000) Characterization and overproduction of the *Escherichia coliappA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. Can J Microbiol 46:59–71

Greiner R, Carlsson N, Alminger ML (2000) Stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by a phytate degrading enzyme of *Escherichia coli*. J Biotechnol 84:53–62

Lee DC, Cottrill MA, Forsberg CW, Jia Z (2003) Functional insights revealed by the crystal structures of *Escherichia coli* glucose-1-phosphatase. J Biol Chem 278:31412–31418

Lim D, Golovan, S, Forsberg CW, Jia Z (2000) Crystal structures of *Escherichia coli* phytase and its complex with phytate. Nat Struct Biol 7:108–113

Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high level. J Mol Biol 26:289–298

Mitchell DB, Vogel K, Weimann J, Pasamontes L, van Loon AP (1997) The phytase subfamily of histidine acid phosphatases: isolation of two genes for two novel phytases from the fungi *Aspergillus terreus* and *Mycoliophthora thermophila*. Microbiology 143:245–252

Mullaney EJ, Ullah AHJ (2003) The term phytase comprises several different classes of enzymes. Biochem Biophys Res Commun 312:179–184

Oh B-C, Choi W-C, Park S, Kim Y-O, Oh T-K (2004) Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol 63:362–372

Pradel E, Bouquet PL (1988) Acid phosphatases of *Escherichia coli*: molecular cloning and analysis of *agp*, the structural gene for a periplasmic acid glucose phosphatase. J Bacteriol 170:4916–4923

Sajidan A (2002) PhD thesis. Humboldt University, Berlin

Sajidan A, Farouk A, Greiner R, Jungblut P, Müller E-C, Borriss R (2004) Molecular and physiological characterisation of a 3-phytase from soil bacterium *Klebsiella* sp. ASR1. Appl Microbiol Biotechnol 65:110–118

Swofford DL (2002) PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, MA, USA

Thompson JD, Higgins D, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Tye AJ, Siu FKY, Leung TYC, Lim BL (2002) Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis*. Appl Microbiol Biotechnol 59:190–197

Zinin NV, Serkina S, Gelfand MS, Shevelev AB, Sineoky SP (2004) Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus*. FEMS Microbiol Lett 236: 283–290