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Expression, glycosylation and secretion of yeast acid phosphatase in hamster BHK cells

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The gene PHO5 coding for one of the repressible acid phosphatases of the yeast *Saccharomyces cerevisiae* has been expressed at high efficiency in the baby hamster kidney (BHK) cell line. The expression vector was constructed from PHO5 driven by the human β-actin promoter and was transfected into BHK cells by the calcium phosphate method. The recombinant APase (r-APase) which was secreted in active form from the cells was estimated by SDS/polyacrylamide gel electrophoresis to have molecular mass $M_r = 62\,000$, indicating substitution of the polypeptide moiety by 2–3 asparagine-linked glycans. Analysis by sequential lectin affinity chromatography of glycopeptides obtained from r-APase with Pronase showed that the glycans are predominantly of the 2.2.4 triantennary and tetraantennary complex-type. These data suggest that the extensive glycosylation of yeast APase, which contains eight polymannose substituents, is not essential for secretion and expression of enzymatic activity of the transfected gene product.

Keywords: yeast acid phosphatase, glycosylation, transfection, BHK cells

Abbreviations: APase, acid phosphatase; PBS, phosphate buffered saline; TBS, Tris buffered saline; con A, concanavalin A; TCA, *Tetracarpidium conophorum* agglutinin.

Acid phosphatase (APase) from the yeast *Saccharomyces cerevisiae* is an extracellular glycoprotein which is secreted into the periplasmic space [1, 2]. One of the biological functions of APase appears to be protein dephosphorylation [3, 4], an important event in enzyme regulation and in protein catabolism. The APase enzyme contains per subunit 8/9 asparagine-linked oligosaccharides [5]. These include five unprocessed or poorly processed chains with 9–14 mannose residues and three chains elongated by the addition of, on average, 48, 92 and 150 mannose residues, respectively. The carbohydrate chains are important mediators of APase secretion and of subunit interactions [6–10]. The active form of APase is an octamer and underglycosylated forms isolated from tunicamycin treated yeast cells are unstable and tend to form inactive or partially active dimers [11].

The overall purpose of the present project aims to establish more fully the role of appropriate glycosylation in the assembly, enzymatic activity and secretion of APase. This paper describes the construction of a vector containing an APase gene and suitable for expression in mammalian cells. We have expressed the APase gene in BHK cells. The enzyme is synthesized and secreted into the medium with high efficiency by transfected BHK cells. The pattern of glycosylation of the secreted enzyme is reported. The availability of a plasmid for highly efficient expression of PHO5 in mammalian cells will be useful for the generation of glycosylation variants of APase using site-directed mutagenesis of the PHO5 gene for expression in BHK cells and by expression in lectin-resistant and glycosylation-defective mutants.

Materials and methods

Materials

Restriction and DNA modifying enzymes were from Boehringer Mannheim, Germany. Sequenase was from US Biochemical Corp, Cleveland, OH, USA. The plasmid pBr322 (HIS3)/PHO3, PHO5 was kindly provided by W. Hörz, Munich. Purified yeast acid phosphatase was obtained as described [2]. Polyclonal antibodies raised against native enzyme or a preparation deglycosylated by endoglycosidase H were obtained by injection of rabbits intradermally with 500 µg of deglycosylated or 1 mg of native protein followed by booster injections intradermally after one month. Sera were taken periodically thereafter. Plasmid pHβA Pr-1-neo was obtained from F. Walsh (Guys Hospital,

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London, UK). [2-^{3}H]Mannose (10.4 Ci mmol^{-1}) was from Amersham International, UK. Protein A Sepharose, concanavalin A Sepharose and lentil lectin Sepharose were from LKB Pharmacia, Milton Keynes, UK. *Tetracarpidium conophorum* lectin (TCA) Sepharose was provided by S. Sato and T. Animashaun (NIMR, London, UK). *Arthrobacter ureafaciens* neuraminidase was from Nacali Tesque, Kyoto, Japan. All other reagents were from Sigma Chemical Co., Poole, UK.

Expression vector of APase

Standard techniques [12] were used to subclone the full length DNA of yeast acid phosphatase PHO5 [13, 14] into the *Bam*HI site of the eukaryotic expression vector pHβA Pr1-neo [15]. The plasmid pBR322 (HIS3)/PHO3, PHO5 [13, 14] containing structural genes for both repressible (PHO5) and constitutive (PHO3) acid phosphatases was used to isolate a 3.3 kb *Bcl*I/*Bam*HI fragment carrying the whole PHO5 gene and part of the PHO3 structural gene. This fragment also includes the PHO5 promoter whose activity in yeast cells is influenced by the inorganic phosphate level in the medium [16]. However, since this regulatory mechanism requires a number of different unlinked yeast gene products [17] which are not present in BHK cells, it is expected that gene expression in these cells should be under exclusive control of the human β-actin promoter. Orientation of the construct was verified by restriction analysis and by sequencing with primer 5'-AGA CGA CGT CCG CTT AC-3' based on a region of the PHO5 upstream DNA sequence (see Fig. 1) using dideoxy sequencing by the Sequenase kit (US Biochemical). Plasmid DNA was purifed by caesium chloride gradient centrifugation. Some preparations were purified further by Gene Clean (Bio 101, La Jolla, CA, USA).

Transformation of BHK cells

Transformation with the expression vector was carried out by the calcium phosphate method [18]. Briefly, BHK cells (approximately 10^8) were grown to sub-confluency in monolayer culture on 9 cm diameter plastic plates in Eagles medium supplemented with 10% foetal calf serum at 37°C. The cells were treated with 10–30 µg plasmid DNA exactly as described by Chen and Okayama [18] overnight at 35°C in a low (30%) CO_2 atmosphere. Medium was removed, cells rinsed with growth medium and grown at 37°C in 5% CO_2 for 1 day, split by trypsinization (1:10) into fresh medium and grown for a further day. Finally, cells were kept in selection medium, growth medium containing 400 µg G418 per ml, for up to two weeks. Surviving colonies were harvested by trypsinization and grown separately to confluency in medium with 50 µg G418 per ml and grown routinely in this medium.

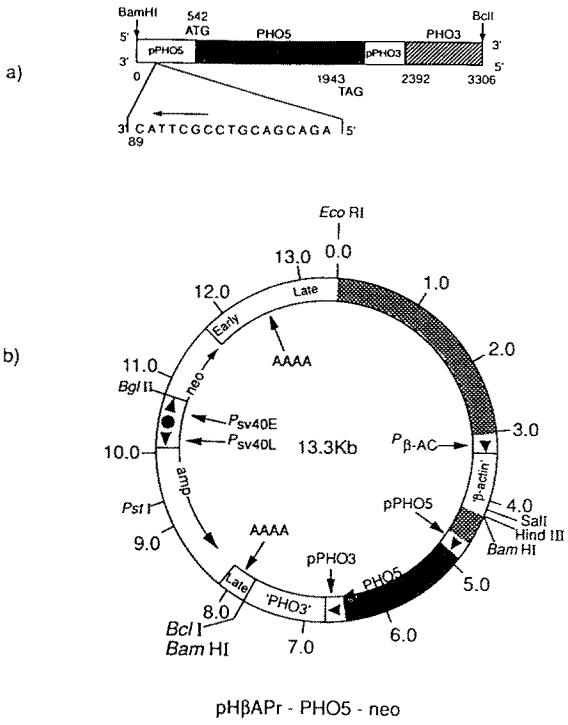

Figure 1. Construction of an expression plasmid containing the yeast acid phosphatase PHO5 gene under the control of the human β actin promoter. (a) Diagram of the PHO5 containing *Bam*HI-*Bcl*I cDNA insert isolated from plasmid pBR322 (H1S3)/PHO3, PHO5. The sequence of the primer used for DNA sequencing analysis is shown. (b) Restriction map of the expression plasmid pHβAPr-PHO5-neo containing the *Bam*HI-*Bcl*I insert subcloned into the single *Bam*HI site of the plasmid pHβAPr-1-neo. The *Bcl*I-*Bam*HI site is a hybrid site as a result of the insertion of the *Bam*HI-*Bcl*I 3.3 kb fragment. The insert also contains a partial PHO3 sequence.

Electrophoresis

SDS/Polyacrylamide gel electrophoresis was performed in 7.5%, 10% or 12.5% gels. Western blot analysis was performed essentially as described by Towbin *et al.* [19]. Protein standards used were myosin, β-galactosidase, phosphorylase b, bovine serum albumin, egg albumin and carbonic anhydrase (MW-SDS-200 Kit, Sigma) or prestained standards of α_2-macroglobulin, β-galactosidase, fructose 6-phosphate kinase, pyruvate kinase, fumarase, lactic dehydrogenase and triosephosphate isomerase (MW-SDS-BLUE Kit, Sigma).

Detection of APase

Monolayer cultures of transfected and nontransfected cells grown in 75 cm^2 flasks to 80% confluency were washed twice with phosphate buffered saline (PBS), covered with Ham's F10 medium without serum and kept at 37°C for 20–24 h. The medium (10 ml) was Millipore filtered, dialysed against 10 mM Tris-HCl, pH 7, and concentrated by lyophilization. The residue was resuspended in distilled H_2O

(100 µl). Proteins separated by SDS/polyacrylamide gel electrophoresis were transferred to nitrocellulose and probed by Western blotting with rabbit anti-yeast APase diluted 1:250 in 5% Marvel, 50 mM Tris HCl pH 7.5, 150 mM NaCl (TBS) followed by alkaline phosphatase-conjugated goat anti-rabbit Ig diluted 1:500. For activity measurements, monolayer cultures were rinsed in PBS and Eagles medium with 10% foetal calf serum and without phenol red (10 ml per 75 cm flask) was added. The cells were kept at 37°C for 20–24 h and the medium was collected, passed through a 0.45 µ Millipore filter and dialysed at 2°C overnight against 50 mM sodium acetate buffer, pH 3.8. The cells were trypsinized and suspended finally in PBS (1 ml). Cell number was measured by optical density at 600 nm. For activity measurements, aliquots of medium (0.2–1 ml) were adjusted to 1 ml with 50 mM sodium acetate, pH 3.8, and added to 20 mM *p*-nitrophenyl phosphate (1 ml). The mixtures were incubated at 37°C for various times (usually 4 h) after which was added 1 M NaOH (3 ml). Optical densities were measured at 400 nm.

Metabolic labelling

Transfected or nontransfected BHK cells growing as monolayers in 9 cm diameter dishes were rinsed with PBS and labelled by incubation for 4–6 h at 37°C with 5 ml Eagles medium containing $\frac{1}{10}$th the normal concentration of glucose supplemented with 10% dialysed serum and [^{3}H]mannose (0.5 mCi per dish). The cells were then chased by replacing the labelling medium with normal Eagles medium supplemented with 5 mM cold mannose (5 ml). After incubation at 37°C for 20–24 h, the medium was collected, Millipore filtered, freeze-dried and redissolved in H_2O (0.5 ml).

Glycopeptide analysis

Aliquots (25 µl) of the concentrated medium obtained from [^{3}H]mannose labelled transfected cells were subjected to SDS/polyacrylamide gel electrophoresis and the separated proteins were located by Coomassie Blue staining. A 5 mm wide gel line containing the APase band was excised with a razor blade, and the gel pieces transferred into Eppendorf tubes. The gel pieces were washed with three 1 ml aliquots each of 25% 2-propanol followed by methanol, air dried and then incubated [20] at 37°C for 24 h with Pronase (0.4 mg ml^{-1}) in 50 mM Tris-HCl, pH 8, 5 mM $CaCl_2$ (0.2 ml). The digests combined from several gels were pooled, concentrated to 1 ml and applied to a column (2 cm × 26 cm) of Bio-Gel P-2. Fractions (2 ml) eluted with H_2O were analysed for radioactivity and peak excluded fractions 21–25 were pooled and freeze-dried. The glycopeptides were dissolved in 1 ml of buffer A (10 mM Tris-HCl, pH 7.5:1 mM $CaCl_2$:1 mM $MgCl_2$:1 mM $MnCl_2$:1 mM $NaCl_2$) and applied to a column (2 ml bed vol) of con A Sepharose and eluted sequentially with buffer A, 10 mM methyl α-glucoside and 500 mM methyl α-mannoside, collecting 1 ml fractions. Selected peak radioactive fractions were pooled, desalted on Bio-Gel P-2, redissolved in buffer A (1 ml) and applied to a column (2 ml bed vol) of lentil lectin Sepharose. The column was washed sequentially with buffer A and 200 mM methyl α-glucoside collecting 1 ml fractions [20]. Selected peak radioactive fractions were desalted, dissolved in buffer A (0.5 ml) and treated with *Arthrobacter ureafaciens* neuraminidase (1 unit) for 24 h at 37°C. Following incubation, the solution was heated at 100°C for 5 min to inactivate the enzyme and applied to a column (1 ml bed vol) of TCA Sepharose. Fractions (1 ml) were eluted at room temperature with buffer A, 10 mM lactose and 100 mM lactose, and finally the column was washed at 37°C with 100 mM lactose as described [21].

Binding of APase to TCA Sepharose

Aliquots (0.1 ml) of growth medium collected over 24 h from transfected cells as described before were mixed with TCA Sepharose beads (0.1 ml) and incubated overnight at room temperature. The beads were washed repeatedly by centrifugation using buffer A, suspended in sample buffer for electrophoresis, boiled for 5 min and the supernatant removed. Equal proportions of the fractions bound and unbound to TCA Sepharose were subjected to SDS/polyacrylamide gel electrophoresis and Western blotting as described before.

Results

The animal cell expression vector which we have used in this work is a plasmid containing the human β-actin gene promoter (pHβApr-1-neo), previously reported [15] to have an efficient promoter activity in several different cell lines. We have subcloned the 3.3 kb *Bcl*I/*Bam*HI fragment of a plasmid containing the PHO5 gene for repressible yeast APase [13, 14] into the *Bam*HI subcloning site of the expression vector. The recombinant plasmid DNA (Fig. 1) was analysed by restriction enzyme mapping and the following results were obtained. (a) Digestion by any unique-site restriction enzyme results in linearization of circular plasmid DNA with a total size 3.3 kb higher than that of the vector DNA itself. (b) Combined digestion by *Eco*RI (unique site) and either *Bam*HI or *Hind*III (which are subcloning sites located on a short synthetic sequence of 44 nucleotides) correctly yields two fragments. (c) *Hind*III/*Bam*HI digestion does not yield a 3.3 kb fragment, indicating that the insert orientation is correct and the *Bam*HI site of the subcloned DNA has remained adjacent to the *Hind*III site. (d) *Sal*I digestion gives a 0.6 kb fragment. This is consistent with the presence of a *Sal*I site in the subcloned DNA at a position approximately 600 bp from the *Sal*I subcloning site. These experiments confirmed that the insertion of the PHO5 DNA fragment was correct and that the vector DNA remained intact. The recombinant DNA construct is 13.3 kb in size and includes the subcloned PHO5

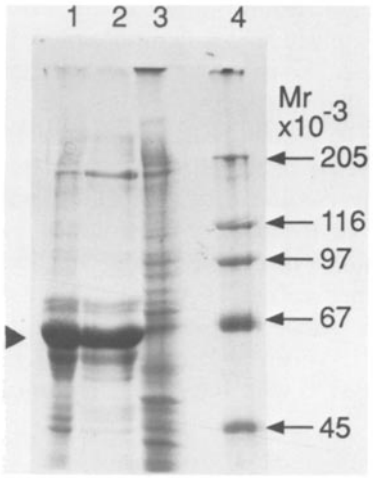

Figure 2. SDS/Polyacrylamide gel electrophoresis of proteins secreted by transfected and nontransfected BHK cells. Medium collected over 24 h from cells transfected with plasmid pHβAPr-PHO5-neo (see Fig. 1) or from control cells was concentrated by lyophilization and equal amounts of protein (approximately 50 µg) were subjected to electrophoresis. Proteins were detected by Coomassie Blue staining. Tracks 1 and 2, separate colonies of transfected cells; track 3, untransfected cells; track 4, protein standards. The prominent band $M_r = 62\,000$ in medium of transfected cells is indicated by the large arrowhead.

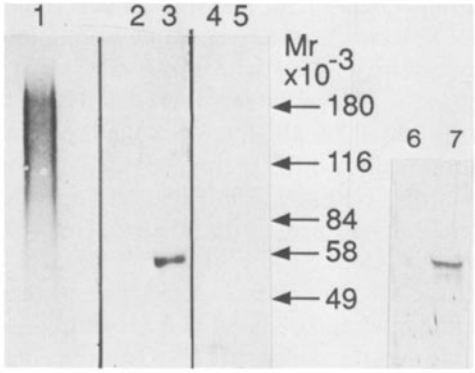

Figure 3. Western blotting of APase. Track 1, yeast APase (approximately 20 µg); tracks 2 and 4, medium collected from control BHK cells over 24 h (approximately 50 µg protein); tracks 3 and 5, medium collected from transfected cells (approximately 50 µg protein). Tracks 1, 2 and 3 were probed with APase specific antibodies; tracks 4 and 5 were probed with nonimmune serum. Protein bands were visualized using alkaline phosphatase conjugated second antibody. The anomalous migration of the $M_r = 58\,000$ protein standard (pre-stained, Sigma) is discussed in the text. Tracks 6 and 7 show an antibody blot of the components secreted by transfected cells that were unbound or bound to TCA Sepharose, respectively.

gene, selectable markers for animal cells (neomycin-resistance gene) and bacteria (ampicillin-resistance gene) and the human β-actin promoter and coding sequences.

This construct (pHβAPr-PHO5-neo) was introduced into BHK cells by a calcium phosphate method [18]. The cells were then selected for resistance to the neomycin analogue G418 for two weeks. Single colonies were isolated, grown to confluence and analysed for acid phosphatase expression. As Fig. 2 shows, the growth medium collected from transfected BHK cells cultured for 24 h at 37°C contained a predominant protein band that was absent in the growth medium obtained from nontransfected cells. In all, 12 separate colonies of transfected cells obtained after selection were examined. Eleven of these gave results very similar to those shown in Fig. 2, tracks 1 and 2. The amount of protein present in the major band was estimated from a standard curve obtained by SDS/polyacrylamide gel electrophoresis of known amounts of bovine serum albumin followed by Coomassie Blue staining. The result indicated that the major protein is present in the growth medium of transfected cells (approximately 5×10^7 cells) at a concentration of approximately 5 µg ml^{-1}. The molecular mass of the protein was calculated to be 62 000 Da by reference to a collection of protein standards.

Direct evidence that the major secreted protein component represents APase was obtained by Western blotting (Fig. 3). The medium from transfected cells contained a single component reacting with antibodies to intact (Fig. 3, track 3) or deglycosylated (results not shown) yeast APase, whereas no immunologically reactive component was present in nontransfected cells (Fig. 3, track 2). Non-immune serum recognized no components in the secretions of either transfected or control cells (Fig. 3, tracks 4 and 5). The molecular mass of the immunologically reactive protein was calculated to be 62 000 Da from the relative migrations of protein standards. It should be noted that the $M_r = 58\,000$ protein pre-stained standard (pyruvate kinase) behaved anomalously in our hands during electrophoresis and was excluded from the calibration curve used to estimate the size of the secreted APase. Figure 3 also shows that APase prepared from yeast migrates during SDS/polyacrylamide gel electrophoresis as a highly disperse component with molecular mass in excess of approximately 100 kDa.

APase produced by transfected BHK cells was shown to be enzymatically active as measured by hydrolysis of *p*-nitrophenyl phosphate at pH 3.8, the pH used routinely for assay of the yeast enzyme. The medium obtained from nontransfected BHK cell cultures showed (Fig. 4) significant hydrolysis of the substrate at pH 3.8, presumably due to lysosomal phosphatase activity working at sub-optimal pH. However, the phosphatase activity measured in secretions obtained from the transfected cells was approximately 2–3-fold greater than in equivalent amounts of material obtained from control BHK cells.

Glycopeptide analysis of the secreted APase showed that the glycans were predominantly of the tri- and tetraantennary complex-type (Fig. 5). When the glycopeptide fraction obtained by gel filtration of Pronase digested APase, labelled

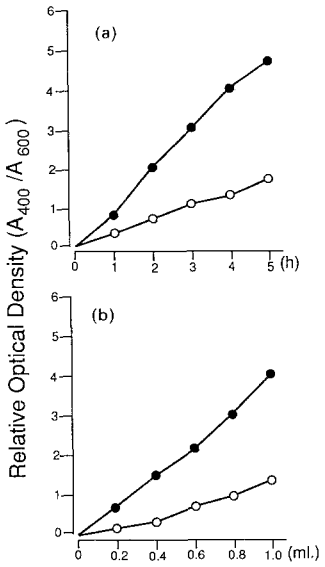

Figure 4. Detection of APase activity in the growth medium of BHK cells transfected with PHO5 containing plasmid. (a) Medium (1 ml) was incubated with *p*-nitrophenyl phosphate at pH 3.8 for various times. (b) Various amounts of medium incubated at pH 3.8 with substrate for 4 h. ●, Transfected cells; ○, nontransfected cells. The results obtained using the two cell lines (transfected and nontransfected) have been normalized to take account of the cell numbers used to collect medium for activity determination. See the Materials and methods section for details.

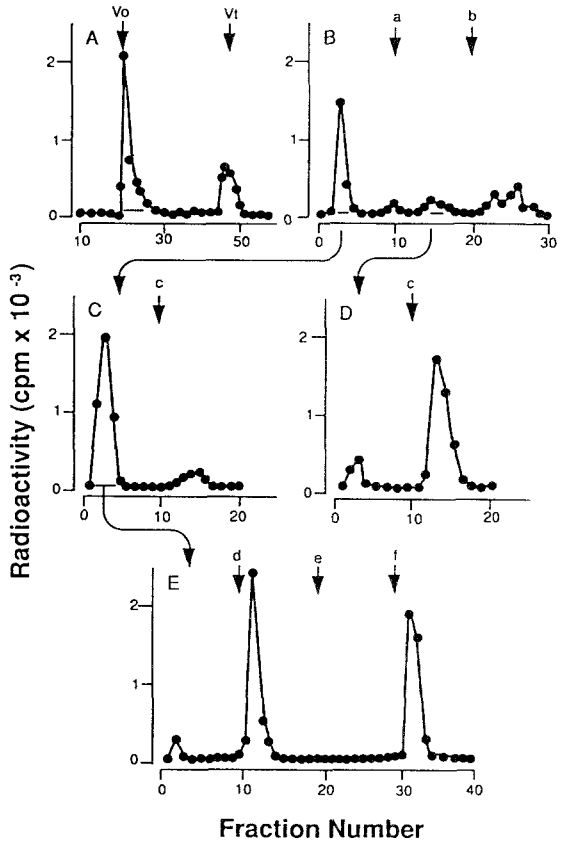

Figure 5. Glycopeptide analysis of secreted APase. APase isolated from the secretions of [^{3}H]mannose-labelled BHK cells was digested with Pronase and the glycopeptides obtained by Bio-Gel P-2 chromatography (A) were fractionated on con a Sepharose (B). Glycopeptides eluted with buffer and with 10 mM methyl α-glucoside were then chromatographed on lentil lectin Sepharose (C, D, respectively). For TCA Sepharose chromatography (E) glucopeptides were first incubated with *Arthrobacter ureafaciens* neuraminidase and then applied to the column. Vo and Vt, void and total volumes. Column elution: a, 10 mM methyl α-glucoside; b, 500 mM methyl α-mannoside; c, 200 mM methyl α-glucoside; d, 10 mM lactose at 20°C; e, 100 mM lactose at 20°C; f, 100 mM lactose at 37°C.

with [^{3}H]mannose, was applied to con A Sepharose, most (70–80%) of the radioactivity was eluted with buffer representing tri/tetraantennary glycopeptides with minor peaks being eluted with 10 mM methyl α-glucoside and 500 mM methyl α-mannoside representing respectively biantennary and oligomannose glycopeptides [20]. Lentil lectin Sepharose chromatography showed that most of the biantennary fraction was core fucosylated, and revealed the presence of a minor amount of core fucosylated 2.2.6 triantennary glycans in the con A Sepharose-unretarded fraction. The fraction unretarded on con A Sepharose and lentil lectin Sepharose gave two approximately equal peaks on TCA Sepharose chromatography. The fraction eluted with 10 mM lactose represents tetraantennary and possibly small amounts of non-fucosylated 2.2.6 triantennary complex-type glycans, whereas 2.2.4 triantennary complex-type glycans are eluted with 100 mM lactose at 37°C. TCA binds with equal facility to core fucosylated or nonfucosylated glycans [21] but, by analogy to the biantennary glycopeptide fraction, it is probable that most of the tri- and tetraantennary glycans carry a core fucose substituent. The complex-type glycans of recombinant APase are exposed since incubation of an aliquot of the growth medium of transfected cells with TCA Sepharose removed APase completely, as judged by Western blotting of the bound and unbound fractions (Fig. 3).

Discussion

The results presented here show that the PHO5 gene coding for yeast and phosphatase has been successfully expressed in BHK cells. The level of expression and secretion attained (approximately 5 mg l^{-1} culture medium) compares favourably with those reported in high producer cell lines for other transfected gene products (4–16 per l).

Expression of the PHO5 gene in BHK cells had a dramatic effect on the glycosylation of the protein product as expected. The enzyme purified from yeast is polydisperse, as shown by SDS/polyacrylamide gel electrophoresis (Fig. 3), due to the presence of a large and variable content of carbohydrate with outer chain polymannose extensions [5, 10]. Mammalian cells lack the processing machinery

required for such modifications [22]. Hence, the product expressed in BHK cells migrated during SDS/polyacrylamide gel electrophoresis as a sharp band with $M_r = 62\,000$. This value together with the calculated size of the polypeptide moiety of APase indicates the presence of two or three asparagine linked oligosaccharides.

Interestingly, the APase secreted by transfected BHK cells exhibits measurable enzymatic activity. Previous work has indicated that outer chain elongation of the oligomannosidic glycans of the yeast APase is not essential for secretion of the active enzyme and that only four glycans out of the eight or nine attached to the protein are sufficient for transport to the periplasmic space [9, 10]. Enzyme derivatives containing fewer than four oligosaccharide substituents by contrast were retained within the endoplasmic reticulum in a poorly soluble, presumably aggregated form. *In vitro*, the underglycosylated APase was found to be less stable than the native form [6–8], suggesting that a minimum level of glycosylation is required for the correct folding of APase into a soluble, polymeric and fully active form. The present results are broadly in agreement with and extend previous findings. Evidently, substitution of the protein with two or three tri- and tetraantennary complex-type oligosaccharides, as in the product synthesized in transfected BHK cells, is sufficient to maintain the enzyme in a functionally active form and to mediate its secretion. However, further work is required to determine the specific activity, oligomeric status and solubility characteristics of the transfected gene product. Additionally, it remains to be determined which of the 8/9 sites glycosylated in the native yeast APase are utilized in the transfected gene product. It has been suggested that the extent to which the oligosaccharide moieties of glycoproteins are processed during biosynthesis depends on the physical accessibility of the glycans to processing enzymes [23]. On this basis and given the fact that the glycans of the transfected APase gene product are predominantly of the tri- and tetraantennary complex type, it seems probable that the sites utilized in the transfected cells normally carry outer-chain elongated polymannose chains in the yeast APase. However, yeast invertase obtained from transfected murine fibroblasts [24] or from oocytes [25] appears to contain complex-type glycans at sites that normally carry largely unprocessed oligomannosidic chains in the yeast-derived enzyme, suggesting that site accessibility is not a limiting factor in oligosaccharide processing, at least in some cases.

In conclusion, the system described here should allow a detailed examination to be made of the roles of glycosylation on APase folding, secretion and stability. APase molecules containing different patterns of glycosylation can readily be obtained by transfection of lectin resistant BHK cell mutants. Of particular interest is the expression of APase DNA clones in which one or more of the sites glycosylated in transfected BHK cells is deleted by site-directed mutagenesis.

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