A NOVEL FORM OF 6-PHOSPHOFRUCTOKINASE: IDENTIFICATION AND FUNCTIONAL RELEVANCE OF A THIRD TYPE OF SUBUNIT IN PICHIA PASTORIS

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Running Title: phosphofructokinase from Pichia pastoris

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Classically, 6-phosphofructokinases are homo- and hetero-oligomeric enzymes consisting of alpha subunits and alpha/beta subunits, respectively. Herein, we describe a new form of 6-phosphofructokinase (Pfk) present in several Pichia species, which is composed of three different types of subunit, alpha, beta and gamma. The sequence of the gamma subunit shows no similarity to classical Pfk subunits or to other known protein sequences. In-depth structural and functional studies revealed that the gamma subunit is a constitutive component of Pfk from Pichia pastoris (PpPfk). Analyses of the purified PpPfk suggest a hetero-dodecameric assembly from the three different subunits. Accordingly, it is the largest and most complex Pfk identified yet. Although, the gamma subunit is not required for enzymatic activity, the gamma subunit-deficient mutant displays a decreased growth on nutrient limitation and reduced cell flocculation when compared to the P. pastoris wild type strain. Subsequent characterization of purified Pfk from wild type and gamma subunit-deficient strains revealed that the allosteric regulation of the PpPfk by ATP, Fru 2,6-P2 and AMP is fine tuned by the gamma subunit. Therefore, we suggest that the gamma subunit contributes to adaptation of P. pastoris to energy resources.

The ATP-dependent 6-phosphofructokinase (EC 2.7.1.11, phosphofructokinase-1, ATP: D-fructose-6-phosphate-1-phosphotransferase; Pfk) catalyzes in many organisms the phosphorylation of fructose-6-phosphate (Fru 6-P) at position 1. The Pfk activity is generally being sensitive to a number of allosteric regulators, e.g. ATP, AMP, NH4+, and fructose-2,6-bisphosphate (Fru 2,6-P2). Therefore, this irreversible reaction is considered to be one of the rate-limiting steps of glycolysis (1-3). Most eukaryotic Pfks are heteromeric enzymes consisting of subunits, which evolved from a single ancestor gene by gene duplication and mutational events (4,5). Specific amino acid residues involved in catalytic and regulatory functions of Pfk from E. coli (6,7) are conserved in yeast and mammalian Pfk genes. In eukaryotes the N-terminal half of a Pfk subunit obviously retained the catalytic function, whereas in the C-terminal half allosteric ligand binding sites have evolved from former catalytic and regulatory sites (4,8,9). This assumption is supported by studies with mutants of Saccharomyces cerevisiae expressing only the α or the β subunit of Pfk. It was demonstrated that one subunit type alone is able to form an enzymatically active Pfk entity in vivo (10,11). Crystallographic analysis showed that an active bacterial Pfk consists of four identical subunits (12,13). No high resolution structure of a eukaryotic Pfk is available yet. But electron microscopic studies with S. cerevisiae Pfk (ScPfk) at 10.8 Å resolution suggested an octameric enzyme assembly (14).

Recently we co-purified a protein component together with the known Pfk α and β subunits.
(15-17) from the methylotrophic yeast *Pichia pastoris*. This unknown protein could only be separated under denaturating conditions and with loss of Pfk activity. Herein, we present the sequence of the co-purified component and describe this new protein as a constitutively bound and regulatory relevant subunit of Pfk from *P. pastoris* (*PpPfk*) and other *Pichia* spp. Based on the molecular mass of the native *PpPfk* and the molar ratio and the molecular mass of the individual subunits we propose an enzyme complex formed of four α, β, and γ subunits.

**EXPERIMENTAL PROCEDURES**

**Yeast strains and growth conditions.** Strains used for isolation of nucleic acids and for analysis of Pfk proteins are summarized in suppl. Table S1. Yeast cells were cultivated in YP medium (1% yeast extract, 2% Bacto Pepton) containing 2% glucose or 0.5% methanol at 30 °C under rotation up to the growth phase as indicated. Minimal medium containing 0.67% yeast nitrogen base, 0.5% ammonium sulfate and 2% glucose supplemented with adenine and amino acids but lacking uracil was used as selective medium. All biochemicals for cell cultivation were purchased from Difco (Becton Dickinson, Sparks, USA) and Invitrogen Life Technologies (Karlsruhe, Germany).

**Preparation of cell-free extract and assays.** Cell-free extract was prepared according to Schwock *et al.* (18). Pfk activity measurement followed basically procedures described elsewhere (16). For kinetic studies a Pfk assay with simultaneous ATP and Fru 6-P regeneration (100 mM imidazole/HCl, pH 6.6, 100 mM KCl, 10 mM MgCl₂, 20 mM potassium phosphate, 0.2 mM NADH, 0.6 mM phosphoenolpyruvate, 8.5 U pyruvate kinase/ml, 7 U lactate dehydrogenase/ml, 1 U fructose-1,6-bisphosphatase/ml; ATP, Fru 6-P, AMP and Fru 2,6-P₂ as indicated) was used (16). A two-state Monod-Wyman-Changeux model was applied to describe the ATP velocity curves under the assumptions: (1) an octameric allosteric mode, (2) AMP and Fru 2,6-P₂ binding to the R-state enzyme only, and (3) ATP serves as substrate (*K₃^{ATP}* ) in a hyperbolic manner, but acts also as allosteric inhibitor (*K₃^{ATP}* ).

For description of the Fru 6-P velocity curves and the dependence of Pfk activity on AMP and Fru 2,6-P₂ concentrations, a generalized Hill equation was used:

\[
ν = \frac{V_0^* (X)}{(1 + (X)/K_X^*)^n} \]

\[
L = m_0 \frac{(1+ [AMP]/K_{RK})^n (1+[Fru 2,6-P2]/K_{Fru2,6-P2}^n)}{(1+ [ATP]/K_{ATP}^T)^n (1+ [AMP]/K_{RK})^n (1+[Fru 2,6-P2]/K_{Fru2,6-P2}^n)} \]

(V: maximum activity; *m₀*: allosteric constant; *K₅^{ATP}* : ATP Michaelis constant; *K₇^{AMP}* : ATP-binding constant of the T-state enzyme; *K₁^{AMP}* and *K₁^{Fru2,6-P2}* : AMP and Fru 2,6-P₂ binding constants of the R-state enzyme).

The kinetic data were fitted to eq. 1 and eq. 2 by non-linear regression analysis applying Sigma Plot 9.0 (Systat Software, Inc., San Jose, USA) that uses the Marquardt-Levenberg algorithm for minimization.

Alcohol oxidase (AOX) activity was measured in a reaction coupled to horseradish peroxidase and the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, Taufkirchen, Germany) in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C according to the company’s technical information.

For determination of protein concentrations, the procedure of Bradford (19) was applied using bovine serum albumin as standard.

**Purification of Pfk from *Pichia pastoris* and protein sequencing.** Pfk was isolated from cell-free extract of *P. pastoris* strain MH458 as described previously (16). N-terminal sequences of polypeptides were determined according to the Edman procedure using the Protein Sequencer 473A (Applied Biosystems, Foster City, USA). Tryptic in-gel digestion and MALDI-MS measurements of the generated tryptic peptides were carried out as described previously (20). The mass spectrometric measurements were performed on a Bruker Reflex MALDI-time-of-flight (TOF) mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an ion gate and pulsed ion extraction. Post source decay
After washing with PBS, the antibody was purified by the manufacturer (Amersham Biosciences). BrCN-activated Sepharose 4B as recommended was eluted with 100 mM citrate buffer, pH 3.0, from the column (Amersham Biosciences). The antibody was fractionated by 50% ammonium sulfate saturation. The precipitated protein was dialyzed against 20 mM sodium phosphate buffer, pH 7.0. 200 µg antigen in complete Freund's adjuvant (0.5 ml final volume) were used for animal immunization. After five weeks the animal was boosted in the same way. Antiserum was fractionated by 50% ammonium sulfate saturation. The precipitated protein was dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and loaded onto a protein-A-Sepharose CL-4B column (Amersham Biosciences). The antibody was eluted with 100 mM citrate buffer, pH 3.0, neutralized with 1 M Tris/HCl, pH 9.0, precipitated with ammonium sulfate, and dissolved in PBS. For affinity purification, purified PpPFK was covalently coupled with BrCN-activated Sepharose 4B as recommended by the manufacturer (Amersham Biosciences). After washing with PBS, the antibody was eluted with 3 M MgCl₂, dialyzed alternate against 155 mM NaCl and PBS, and stored at –20 °C. Protein concentration was calculated according to

\[ A_{\text{280nm}}^{\text{cm,mg/cm}} = 1.35 \]  

(PSD) fragment ion spectra were obtained by using the FAST method (Bruker Daltonik).

**Generation of a polyclonal antibody against the γ subunit of PpPFK.** Subunits of purified Pfk from *P. pastoris* strain MH458 were separated under reducing conditions by SDS-PAGE. The γ subunit was cut out, destained in 10% acetic acid containing 40% methanol at 4°C and extracted by electroelution (Electro-Eluter Model 422, BioRad). Then, the protein was dialyzed against phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM NaCl, pH 7.0). 200 µg antigen in complete Freund’s adjuvant (0.5 ml final volume) were used for rabbit immunization. After five weeks the animal was boosted in the same way. Antiserum was fractionated by 50% ammonium sulfate saturation. The precipitated protein was dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and loaded onto a protein-A-Sepharose CL-4B column (Amersham Biosciences). The antibody was eluted with 100 mM citrate buffer, pH 3.0, neutralized with 1 M Tris/HCl, pH 9.0, precipitated with ammonium sulfate, and dissolved in PBS. For affinity purification, purified PpPFK was covalently coupled with BrCN-activated Sepharose 4B as recommended by the manufacturer (Amersham Biosciences). After washing with PBS, the antibody was eluted with 3 M MgCl₂, dialyzed alternate against 155 mM NaCl and PBS, and stored at –20 °C. Protein concentration was calculated according to

\[ A_{\text{280nm}}^{\text{cm,mg/cm}} = 1.35 \]  

**Cloning of PpPFK3 encoding the γ subunit.** Touchdown PCR was carried out with genomic DNA as template and HotStarTaq™ DNA Polymerase (Qiagen, Hilden, Germany). Furthermore, degenerate primers 4 and 14 were used, which corresponded to the identified amino acid sequences of the γ subunit (all primers are listed in suppl. Table S2). PCR was performed under the following conditions: Pre-denaturation at 95 °C for 15 min was followed by cycles of denaturation at 94 °C for 30 s, annealing beginning at 72 °C for 30 s, and elongation at 72 °C for 90 s. The annealing temperature was lowered 1 °C per cycle to 50 °C, which then was applied for annealing in the next 20 cycles. To identify the 5’ and 3’ ends, RACE- (rapid amplification of cDNA ends) PCR was performed as described previously (17) and accordingly to the manufacturer’s protocol (GeneRacer™ Kit with cloned AMV RT; Invitrogen Life Technologies). PCR fragments were subcloned into pCR2.1 (TOPO™TA Cloning™ Kit for Sequencing, Invitrogen Life Technologies) and sequenced in both directions using the ABI PRISM® BigDye™ Terminators v2.0 Cycle Sequencing Kit (Applied Biosystems).

**Generation of individual Pfk subunit-deficient *P. pastoris* strains.** Pfk subunit-deficient *P. pastoris* strains were generated by homologous recombination. Each plasmid (pAE27, pAE28, and pAE34) harbored one of the *PpPFK* genes interrupted by *URA3* from *P. pastoris* (plasmids are depicted in suppl. Fig. S1). Transformation of the *P. pastoris* strain JC307 his4 *ura3* was performed by electroporation (*P. pastoris* adjustment, GenePulser Xcell, BioRad, Munich, Germany). To screen mutants and to verify homologous recombination, Southern blot analyses were performed as described previously (22).

**SDS-PAGE/Western blot analysis.** Western blot analysis followed the description of Bär *et al.* (23) with the exception of the use of 10% polyacrylamide gels. Polyclonal rabbit antibodies against the γ subunit of *PpPFK* (this work) and against the purified ScPfk (24) were applied. The anti-ScPfk antibody showed strong cross-reactivity to the α and β subunits of *PpPFK*. Immunodetection was performed with anti-rabbit-IgG peroxidase conjugate (Dianova, Hamburg, Germany) and Chemiluminescent detection reagents (ECL™ Western Blotting System (Amersham Bioscience-GE Healthcare, Munich, Germany)).

**Fluorescence-activated cell sorting (FACS) analysis and immunofluorescence microscopy.** Cells were characterized by FACS analysis using forward light scatter (FSC) and side light scatter (SSC), reflecting cell size and cell complexity, respectively. These were recorded on linear scales. Flow cytometric analysis was performed using the FACSscalibur™ Scanner equipped with the CellQuest™ software (both Becton-Dickinson, Franklin Lakes, USA). Thus, cells were grown in YP medium containing 2% glucose to an optical density (OD) of approximately OD₅₈₀nm≈10 and diluted 1:51 (v/v) in the respective medium. For analysis of the subcellular distribution of Pfk protein by immunofluorescence microscopy, *P. pastoris* cells (OD₅₈₀nm≈1) were processed according to Pringle *et al.* (25). Polyclonal antibodies against Pfk subunits (see SDS-PAGE/Western blot analysis) were used for specific protein detection. Cy3-labeled goat anti-rabbit-IgG antibody (Dianova, Hamburg, Ger-
RESULTS

Molecular identification of a Pfk γ subunit in P. pastoris.
The protein band corresponding to the unknown polypeptide chain, which was co-purified together with the α and β subunits of PpPfk, was isolated from SDS-polyacrylamide gel. Then, the N-terminal amino acid sequences of this protein and of several fragments obtained by chymotrypsin or trypsin degradation were determined by Edman procedure and MALDI-TOF PSD analysis. Amino acid sequences identified are summarized in Table 1. Based on these results, degenerate primers were designed (suppl. Table S2). PCR and cloning techniques (see Experimental Procedures), revealed a genomic DNA fragment of 3,113 bp containing a complete coding sequence (CDS) of 1,056 bp (GenBank Accession Number AY686600). The transcription start was found at –43 bp from start ATG by 5' RACE-PCR from mRNA. The CDS, further referred to as PpPFK3 (according to the nomenclature of other Pfks), encodes a polypeptide with a predicted molecular mass of 40.8 kDa (Fig. 1). N-terminal and internal amino acid sequences of the co-purified component determined by protein sequencing were identical to the respective sequence regions of the translated open reading frame (ORF) of PpPFK3. However, the potential γ subunit appears at approximately 34 kDa when purified PpPfk is analyzed by SDS-PAGE (see Fig. 5). The integrity of the N-terminal and C-terminal ends of the γ subunit isolated by SDS-PAGE (Table 1) was verified by Edman and MALDI-TOF analyses, respectively. Therefore, proteolytic modifications of this protein can be excluded. Accordingly, the discrepancy between the sequence-based calculated mass and the apparent molecular mass found by SDS-PAGE is caused by the specific migration property of the γ subunit in this electrophoresis.

Extensive sequence analyses with various bioinformatic tools (NCBI blast analyses, Predict-Protein, ELM) revealed no significant sequence similarity to any sequence deposit in GenBank (last analysis 27.02.2007). Within the cloned genomic 5’ non-coding region (approximately 2,000 bp) we identified putative consensus sequences for GCR1. This transcription factor is involved in specific regulatory mechanisms for glycolytic gene expression (26,27). In addition, an incomplete ORF of a hypothetical protein was detected. It shows sequence homology to the hypothetical proteins CAHO2429.1 and DEHAOC03872.g from Kluyveromyces lactis (8/324 = 27%) and from Debaryomyces hansenii CBS767 (111/311 = 35%), respectively.

Screening other yeasts for the presence of the Pfk γ subunit.
To address the question whether the γ subunit is unique to P. pastoris, several other yeasts (suppl. Table S1) were initially screened for immunocross-reactivity by Western blot analysis. For this purpose, we used the polyclonal antibody against the PpPfk γ subunit. Whereas different P. pastoris strains and several other Pichia species displayed an immunoreactive band between 34 kDa and 42 kDa, all extracts of distantly related yeasts (see suppl. Table S1) showed no specific immunoreactivity (data not shown). Next, PCR was applied to amplify ortholog sequences using degenerate primer sets designed on the basis of the γ subunit sequence from P. pastoris strain MH458. So far, the presence of a γ subunit was verified in P. pastoris strains JC307, GS115 and in P. pseudopastorisi (GenBank Accession Numbers DQ352840, DQ374390, DQ386148). The γ subunits from P. pastoris strains JC307 and GS115 are identical and show 95.4% amino acid identity to the γ subunit of P. pastoris strain MH458. The γ subunit of P. pseudopastorisi, a
species closely related to *P. pastoris* (28), displays 79.8% identity at the amino acid level to the γ subunits from *P. pastoris* strains.

**Generation and functional characterization of γ subunit-deficient *P. pastoris* strains.** To analyze the relevance of the γ subunit, three *P. pastoris* mutants were generated by deletion of *PpPFK3*. Correct recombination and gene deletion were confirmed by Southern and Western blot analyses (suppl. Fig. S2). The transformed recipient *P. pastoris* strain, which contained the *Ura3* marker gene homologously integrated at the endogenous *ura3* locus but still maintained an intact *PpPFK3* locus, served as proper control (further referred to as wild type strain).

First, basic cell functions of the three γ subunit-deficient strains were studied. Since these strains behaved identically in all experiments, data are exemplarily shown for JC307-22Δ*pPFK3* (further referred to as γ subunit-deficient strain). Growth of the γ subunit-deficient strain was significantly reduced by 20% after 22 h of cultivation (Fig. 2A). This effect was found also under cultivation in a continuously oxygenized atmosphere. Deletion of the γ subunit did not interfere with the specific Pfk activity in cell-free extracts (Fig. 2B). Wild type and γ subunit-deficient strains were able to grow on medium containing glycerol, rhamnose or cycloheximide. This was determined by cultivation on pre-made culture plates (ID32C bioMerieux system) at 30 °C for 4 days (data not shown). Temperature sensitivity and NaCl tolerance were also indistinguishable between the strains (suppl. Fig. S3).

Further, we analyzed the adaptation ability of the mutant and wild type strains to glucose-containing medium after cultivation on methanol. In both cases a 50% reduction of AOX activity over three hours was observed (suppl. Fig. S4A) as result of induced degradation of peroxisomes (microautophagy) (15, 29). This was accompanied by a 50% increase in Pfk activity (suppl. Fig. S4B).

Further, the strains were tested for endocytosis and vacuolar morphology by in vivo labeling (30). For this purpose we used the fluorescent lipophilic dye FM1-43 (Molecular probes, Brussels, Belgium). FM1-43 internalization from the plasma membrane into endosomes and vacuolar membranes was indistinguishable between mutant and wild type strains (suppl. Fig. S5).

Although, most of the phenotypes of the wild type and the γ subunit-deficient strains are very similar, cells of the wild type strain show remarkable flocculation with increasing cell density. The cells start to adhere at the middle log growth phase. The formed macroscopic flocs rapidly sediment when continuous shaking is stopped. This phenotype is greatly reduced in γ subunit-deficient strains (Fig. 3). We observed that cell-cell adhesion in the wild type strain is abolished in presence of 2 mM EDTA but occurs again after the addition of 5 mM Ca²⁺. Adhesion of cells lacking the γ subunit is also induced by addition of Ca²⁺ but to a lesser extent (data not shown). Differences in the cellular structure of the γ subunit-deficient and the wild type strains were also reflected by FACS analysis (suppl. Fig. S6).

**Kinetic properties of the wild type and γ subunit deficient *PpPfk*s**

Because the γ subunit is not essential for the catalytic function of *PpPfk* per se (see above), we initiated an in-depth kinetic analysis of *PpPfk*s purified from wild type and γ subunit-deficient strains (Tables 2, 3).

The purity of all enzyme preparations was verified by SDS-PAGE (suppl. Fig. S7). In contrast to *ScPfk* (for comparison see (31)) the sensitivity of both *PpPfk*s to AMP (*K₄AMP*) is higher, whereas that to Fru 2,6-P₂ (*K₄Fru₂₆-P₂*) is about 10-fold lower. Furthermore, the two *PpPfk*s forms are characterized by a more potent ATP inhibition (residual activity 0f 0.53 ± 0.05 for *ScPfk*). Comparing the kinetic data of the wild type and the γ subunit-deficient *PpPfk*s, no remarkable differences in the half-saturating Fru 6-P concentration (*K₄Fru₆-P*) and the Fru 6-P cooperativity (*n₅H*) in presence and absence of AMP and Fru 2,6-P₂ were found. Further, Michaelis constants (KₛₐT, Table 3) for ATP as substrate are equal. However, a lower affinity of the allosteric ATP-binding site (KₛₐT, Table 3) was determined for the γ subunit-deficient *PpPfk*. Consequently, this enzyme form shows a less efficient inhibition by ATP (suppl. Fig. S8). Further, a remarkable lower sensitivity of the mutated *PpPfk* to AMP was observed. As shown in Figs. 4A, C, the reduced AMP activation becomes apparent especially at physiological ATP levels (>1 mM (32)). The γ subunit-deficient *PpPfk* is also less sensitive to Fru 2,6-P₂ at intermediate ATP concentrations (Figs. 4B, D). However, with increasing ATP levels the Fru 2,6-P₂ activation ratio of the two enzymes converges (Fig. 4B). At very high concentrations (about 5 mM ATP) the activation
ratio of the γ subunit-deficient PpPfk exceeds that of the wild type enzyme (data not shown). In terms of the Monod-Wyman-Changeux model (eq.1), differences in kinetics of the two PpPfk forms could not be described by changes of the allosteric constant (m0). However, the data fitting revealed differences in the constants characterizing ATP binding to the T-state enzyme and AMP and Fru 2,6-P2 binding to the R-state enzyme (Table 3).

**Interactions and assembly of PpPfk subunits.** Recent studies have shown that the extent of proteolytic degradation depends on both, the specificity of the protease and the presence of protective substrates or allosteric effectors such as ATP (33). Therefore, limited proteolysis of the purified PpPfk was performed with chymotrypsin in presence of saturating ATP concentration to analyze the topology of the subunit assembly (detailed conditions are given in the legend of Fig. 5). After proteolysis, enzyme activity was reduced by only 10% in comparison to the non-degraded PpPfk. Although the three subunits possess multiple cleavage sites to chymotrypsin, only the β subunit (~70 kDa, β') and γ subunit (~20 kDa, γ') were truncated as demonstrated by SDS-PAGE (Fig. 5, lane 3). The identity of the γ' fragment was verified by Edman protein sequencing (Table 1). To analyze whether the chymotrypsin-treated enzyme still forms a high molecular complex, the sample was subjected to HPLC gel filtration. A main protein fraction was obtained, which corresponded to approximately 900 kDa (data not shown). The analysis of this 900 kDa-protein fraction by SDS-PAGE (Fig. 5, lane 4) revealed a band pattern identical to the non-fractionated chymotrypsin-treated PpPfk (Fig. 5, lane 3).

Immunoprecipitation was carried out to further prove interactions between the three different PpPfk subunits. As shown in Fig. 6, α and β subunits were only precipitated with the γ subunit-specific antibody in presence of the γ subunit (wild type strain). Although precipitation was not complete, the relative signal intensities of α and β subunits were always equal in the supernatant and in the protein-A-precipitated fraction. This result is indicative for a defined stoichiometry between the subunits.

Further, wild type and γ subunit-deficient strains were subjected to immunofluorescence microscopy to analyze subcellular distribution of PpPfk. The three different subunits displayed a similar cytosolic distribution pattern (suppl. Figs. S9A, B). The subcellular distribution of the α/β subunits remained unchanged in the γ subunit-deficient strain (suppl. Fig. S9C).

To analyze whether the γ subunit can exist independently from the classical Pfk subunits, α and β subunit-deficient strains were generated by homologous recombination. The β subunit encoding gene, PpPFK2, was cloned recently (17), but sequence information of PpPFK1, encoding the PpPfk α subunit, was lacking. We isolated a 6,862-bp genomic fragment containing the complete CDS of PpPFK1 (2,970 bp) and parts of the 5' and 3' non-coding regions (3,809 bp and 83 bp, respectively) (GenBank Accession Number AF508861; suppl. Fig. S10) from *P. pastoris* strain MH458. Homologous recombination of the constructs pAE27 (PpPFK1) and pAE28 (PpPFK2) (suppl. Fig. S1) was confirmed by Southern blotting (suppl. Fig. S11). As shown in Fig. 7A (lane 2), deletion of the α subunit resulted in nearly loss of β and γ subunits in the cytosolic fraction. Analyzing the cell-free extract of the β subunit-deficient strain, we found the α subunit but only a very low amount of the γ subunit (Fig. 7A, lane 3). The individual deletion of both, the α subunit and the β subunit, significantly retarded yeast growth on glucose and abolished Pfk activity measurable in cell-free extract (Fig. 7C, lanes 2, 3).

Reconstitution of the wild type PpPfk from the individual subunits should provide further evidence for association of the γ subunit with the α/β complex. As stated above, the γ subunit can only be separated from purified PpPfk under denaturating conditions (16). Therefore, we initially attempted to reconstitute the enzyme from the individual subunits following complete denaturation by urea or guanidine hydrochloride. In contrast to Pfk from *S. cerevisiae* (34), all efforts failed to refold and assemble the individual subunits. Next, we tried to express the γ subunit in *S. cerevisiae* in order to reconstitute the purified polypeptide with the isolated α/β complex from PpPfk. Heterologous expression attempts were carried out under the control of the original PpPFK3 promoter region and of the promoter of the ScPfk β subunit. The wild type and His-tagged γ subunit constructs yielded only very low quantities of protein. The expression product was also sensitive to proteolytic degradation and aggregated during purification (data not shown).

Finally, we performed reconstitution experiments with the PpPfk subunit deletion mutants according to Klinder et al. (11). Thus, two mutants individually lacking α and β, α and
γ, or β and γ subunits were mixed equally prior cell disruption. PpPfk activity was measured in cell-free extracts of these three strain combinations (Fig. 7C). The Fru 2,6-P2 activation was also monitored at 5 mM and 0.3 mM ATP (Fig. 7D), because at ATP concentrations >3 mM the activation rate of the γ subunit-deficient PpPfk is higher compared with the wild type PpPfk. As expected, individual deletion of either the α subunit or the β subunit is associated with loss of measurable PpPfk activity (Fig. 7C). However, after mixing the cells of these mutants followed by combined cell-disruption, residual Pfk activity was measured in the resulting extract. Moreover, the restored activity was slightly increased by addition of Fru 2,6-P2 (Fig. 7D). As mentioned above, the γ subunit is not essential for the catalytic function of PpPfk. But its deletion alters the PpPfk sensitivity e.g. to Fru 2,6-P2 (Figs. 7C, D). The native PpPfk phenotype in respect of Fru 2,6-P2 activation was restored in the extract of mixed γ subunit-deficient cells and cells lacking the α or the β subunit (both expressing the γ subunit) (Fig. 7D). To confirm a PpPfk assembly from the three subunit types, the cell-free extracts of the mutant combinations were subjected to immunoprecipitation with the γ subunit-specific antibody. Immunoprecipitates were analyzed by Western blotting using the anti-α/β subunit antibody (Fig. 7B). As shown in Fig. 7B (lane 6), an efficient α/β/γ complex assembly was obtained in the extract of mixed α subunit- and γ subunit-deficient cells. The other two mutant combinations revealed only faint signals for the precipitated α/β subunits. This is probably the result of the already low amount of the remaining PpPfk subunits in the respective mutant strains (Fig. 7A). Further, one can speculate that the γ subunit is stabilized by the β subunit but not by the α subunit. Consequently, the stabilized γ subunit can more efficiently assemble with the high amount of the functional α/β complex preformed in the γ subunit-deficient cells. To verify the complexation of α, β and γ subunits, cell-free extracts of the mutant combinations were fractionated by HPLC gel filtration. Pooled fractions corresponding to the molecular mass of the wild type PpPfk were concentrated and analyzed by Western blotting. Similar to the wild type enzyme (suppl. Fig. S12A), the extract of mixed α subunit- and γ subunit-deficient cells showed α/β and γ subunit-specific immunoreactivity (suppl. Fig. S12B).

DISCUSSION

Pfk from Pichia consist of three different subunits.

All known pro- and eukaryotic ATP-dependent Pfks are, so far, homo- or hetero-multimeric enzymes, which assemble from α subunits and α/β subunits, respectively. Herein, we demonstrate that Pfk from Pichia species displays an even more complex structure. We have identified and sequenced a new protein component, which was co-purified together with the α and β subunits of PpPfk (Fig. 1). Further, we provided strong structural and functional evidence that this 40.8-kDa protein is indeed a constitutive third subunit type of PpPfk. First, using an antibody against the γ subunit, the α and β subunits of PpPfk were co-precipitated in a stoichiometric manner (Fig. 6). Second, partial digestion of the purified native enzyme with chymotrypsin led to truncated β and γ subunits, which still form a stoichiometric complex with the α subunit (Fig. 5). Third, immunofluorescence studies showed identical subcellular distributions for the α/β subunits and the γ subunit (supp. Fig. S9). Fourth, clear evidence of association came from the gene deletion studies, which demonstrated that the presence of the γ subunit in the cytosolic fraction critically depends on the expression of the α and β subunits (Fig. 7A). Interestingly, the lack of the α subunit diminishes the cytosolic presence of both, the β and γ subunits, indicating a subsequent dependence on each other. Finally, further evidence that the γ subunit is a relevant part of PpPfk came from reconstitution experiments (Fig. 7) and functional analyses showing modulator properties of the γ subunit on Pfk activity (see below). In all our investigations, we found no indication that the γ subunit can exist as a free soluble protein not bound to α/β subunits.

The γ subunit displays no sequence similarity to known Pfks subunits and to any other proteins identified yet. Although this protein was not found in the sequenced genomes of S. cerevisiae, K. lactis, S. pombe, and C. albicans, we also identified the γ subunit in P. pseudopastoris (Fig. 1). This is indicative of its distinct presence in at least some Pichia species. Usually, gene duplication events are accounted for the evolutionary occurrence of α and β subunits in eukaryotic organisms (5,8). A similar mechanism can be excluded for the introduction of the γ subunit in Pichia. Therefore, other mechanisms such as lateral gene transfer events.
associate and form large oligomeric complexes. SDS-PAGE. Mammalian Pfks tend to self-fraction when the subunits are arranged in the enzyme complex. In contrast to the subunits are probably located inside the native PpPfk.

The γ subunit is involved in adaptation to environmental changes.

The dependence of the glycolytic flux on carbon source and on species-specific requirements was analyzed in various yeasts (45-47). Additionally to the regulation of transcription and posttranslational modification, the allosteric modulation of Pfks and of several other enzymes appears to play the most important role in controlling the glycolytic flux (48-52). However, cells of the methylotrophic actinomycete Amycolatopsis methanolica possess a single inorganic pyrophosphate-dependent Pfk when grown on glucose. However, an ATP-Pfk is induced during cultivation on one-carbon compounds e.g. methanol (53). Based on the unique PpPfk structure suggested above, one can speculate that the existence of a particular Pfk species may be of advantage for methanol-assimilating yeasts such as Pichia. In many organisms Fru 2,6-P2 has been proposed to be the predominant effector of glycolysis (54-56). However, its effect on PpPfk is less pronounced and requires higher metabolite concentrations in comparison to ScPfk (31). It has been suggested that Pfks and other enzymes of the glycolysis can form a complex with aldolase (EC 4.1.2.13) to allow for efficient substrate channeling (57-59). Although γ subunit has no sequence similarities with known aldolases we tested whether the PpPfk containing the γ subunit displays some aldolase activity. We found no aldolase activity by using the coupled enzyme assay (16) without aldolase as auxiliary enzyme where either Fru-6-P or Fru 1,6-P2 served as substrates (data not shown).

To evaluate the functional relevance of the γ subunit, the respective coding sequence was deleted in P. pastoris. The comparison of the wild type and γ subunit-deficient PpPfks (35-37) have to be considered but the origin of the PFK3 sequence remains open.
revealed that the γ subunit is not essential for catalytic function but significantly modulates enzyme kinetics. The γ subunit deficiency is associated with a lower apparent affinity of the regulatory ATP-binding site. Consequently, the ATP inhibition of the mutated PpPfk is less efficient. Further, the γ subunit-deficient PpPfk is characterized by a lower sensitivity to AMP and Fru 2,6-P₂. These results suggest that the sensitivity of PpPfk to ATP inhibition and its reverse by Fru 2,6-P₂ and AMP is fine-tuned by the γ subunit. Our data support the assumption that cellular glucose metabolism in P. pastoris is mainly controlled by ATP and AMP via regulation of PpPfk activity. Atkinson and co-workers proposed in the ‘energy charge’ hypothesis, that most branch points between anabolism and catabolism might be controlled by AMP, ADP and ATP (60) Referring to PpPfk, glycolysis can be throttled by reducing PpPfk activity in the situation of plenty of ATP even in the presence of Fru 2,6-P₂. The higher ATP sensitivity of the γ subunit-containing PpPfk may be of advantage under competitive conditions. In a situation of ATP depletion, PpPfk is efficiently activated by the accompanied increased level of AMP resulting in an enhanced glycolytic flux. Precise regulation of Pfk activity during cellular adaptation to changes in natural carbon sources is particularly important in methylotrophic yeasts like P. pastoris. They are often found in pectin-rich environments such as fruit surfaces containing methyl ester compounds (61). Adaptation to environmental changes, in its extreme to stress, can lead to yeast cell adhesion and formation of macroscopic flocs protecting cells in the centre (62). Flocculation often occurs upon nutrient limitation during late-exponential or stationary phase of growth, and depends on pH, ethanol levels or the carbon source available in the growth medium (63-65). Likewise, P. pastoris displays flocculation in exponential and stationary growth phase (Fig. 3). Interestingly, we found that γ subunit deficiency diminishes cell adhesion. The link between the disturbed flocculation phenotype and the γ subunit is not solved, yet. Flocculation is conferred by adhesins that bind sugar residues and specific peptides or increase the cell-surface hydrophobicity. Expression of these special cell wall proteins is under tight control by several interacting regulatory pathways (62). One can speculate that suboptimal Pfk function due to γ subunit deficiency somehow interferes with proper function of some of these cell surface components. However, it is very unlikely that the γ subunit directly participates in mediating flocculation as described for glyceraldehyde-3-phosphate dehydrogenase from the yeast K. marxianus (66). Adhesion of γ subunit-deficient cells can still be induce by addition of Ca²⁺ but to a lesser extent compared to the wild type strain (data not shown).

In sum, the hetero-dodecameric PpPfk is the most complex Pfk described yet. The newly identified γ subunit is involved in fine-regulation of the enzymatic activity and yeast flocculation. Deletion of the γ subunit reduces yeast growth, as a key marker of cellular fitness. Both, sensitive tuning of Pfk activity and flocculation, appear to be relevant for fitness of Pichia cells. The γ subunit improves these properties and probably provides an advantage for adaptation to environmental changes.

REFERENCES

6. Evans, P. R., and Hudson, P. J. (1979) Nature 279(5713), 500-504
7. Schirmer, T., and Evans, P. R. (1990) Nature 343(6254), 140-145

16. Evans, P. R., and Hudson, P. J. (1979) Nature 279(5713), 500-504

**FOOTNOTES**

**Acknowledgment**

We would like to thank Pietro Nenoff (Laboratory of Medical Microbiology, Moelbis) for biochemical differentiation of *P. pastoris* wild type and γ subunit-deficient strains as well as Mike Francke (Paul Flechsig Institute, Leipzig) for supply of lipophylic dye FM1-43. We are grateful to the anonymous reviewers for the very constructive comments and for many suggestions. We thank Klaus Huse (Fritz-Lipmann Institute, Jena), and Michael McLeish (University of Michigan, Ann Arbor) for critical reading of the manuscript. This work was supported by the IZKF-Leipzig and the Bundesministerium für Bildung und Forschung.
Abbreviations
AOX, alcohol oxidase; CDS, coding sequence; DAPI, 4’,6-diamidino-2-phenylindol; DIG, digoxigenin; FACS, fluorescence-activated cell sorting; FSC, forward light scatter; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometer; OD, optical density; ORF, open reading frame; Pfk, 6-phosphofructokinase; PMSF, phenylmethylsulfonyl fluoride; PpPfk, 6-phosphofructokinase from Pichia pastoris; PSD, post source decay, RACE-PCR, rapid amplification of cDNA ends-polymerase chain reaction; ScPfk, 6-phosphofructokinase from Saccharomyces cerevisiae; SSC, side light scatter; YP, yeast extract (BactoPepton)

Supporting Information
Suppl. Tables S1-S2
Suppl. Figs. S1-S12
Fig. 1. Molecular identification of Pfk γ subunits in different Pichia species.
The coding sequence of the γ subunit of Pfk, PFK3, was cloned from P. pastoris strains MH458, JC307, GS115 and from P. pseudopastoris strain Y01541 (GenBank Accession Numbers AY686600, DQ352840, DQ374390, DQ386148). The isolated sequences from P. pastoris strains GS115 and JC307 are identical. Amino acid sequences of the γ subunit orthologs of P. pastoris strains JC307 and MH458 and from P. pseudopastoris strain Y01541 are shown and conserved positions are indicated (asterisks). Fragments identified by direct protein sequencing of the purified γ subunit from P. pastoris strain MH458 (Table 1) are highlighted. A chymotrypsin cleavage site identified by a limited proteolysis experiment is arrowed.

Fig. 2. Growth curves and Pfk activity of wild type and γ subunit-deficient P. pastoris strains.
Wild type (open square) and γ subunit-deficient (closed square) strains were cultivated in YP medium containing 2% glucose at 30 °C and 250 rpm. Cell density (OD 580nm; A) and specific Pfk activity (B) were determined spectrophotometrically (see Experimental Procedures). Data are given as mean ± S.D. of at least three independent experiments each performed in duplicate.

Fig. 3. Reduced flocculation phenotype of P. pastoris cells lacking the γ subunit of Pfk.
Wild type and γ subunit-deficient strains were cultivated in YP medium containing 2% glucose at 30 °C and 250 rpm up to OD 580nm ≈10. Sedimentation by gravity is depicted at 1 min and at 2 min after taking the samples from shaker.

Fig. 4. Effect of ATP, AMP and Fru 2,6-P₂ on the activity of Pfks from P. pastoris wild type and γ subunit-deficient strains.
ATP-dependent activation of PpPfk from wild type (closed circles) and γ subunit-deficient (open circles) strains was determined at 1 mM AMP (A) and at 20 µM Fru 2,6-P₂ (B). In both experiments, Fru 6-P concentration was kept constant at a physiological level of 0.3 mM. At ATP levels >1 mM, no decrease of the Fru 2,6-P₂ activation ratio is obvious for the γ subunit-deficient PpPfk due to its less efficient ATP inhibition when compared to the wild type PpPfk. The figures are based on the data shown in suppl. Fig. S8. The activation ratio for AMP (C) and Fru 2,6-P₂ (D) was calculated from the enzyme activity in presence (Vₓ) and absence (V₀) of the respective activator (x) at constant Fru 6-P (0.3 mM) and ATP (3 mM) levels. The mutated PpPfk is less sensitive to both, AMP and Fru 2,6-P₂. Data of one enzyme preparation are exemplary shown.

Fig. 5. Limited proteolysis of purified PpPfk.
Purified PpPfk (150 µg) was dissolved in 50 mM sodium phosphate buffer (200 µl, pH 7.0). 2 µl α-chymotrypsin (0.2 mg/ml) and 5 mM ATP were added and the sample was incubated at 25 °C for 2 h. Proteolytic activity was stopped by addition of PMSF (1 mM final concentration) and the sample was divided into two aliquots. One aliquot was kept for SDS-PAGE and the other aliquot was subjected to gel filtration on a SE-HPLC BioSelect SEC400 column (BioRad) using 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. Gel filtration yielded a main protein fraction of ~900 kDa. This fraction was concentrated and analyzed by SDS-PAGE (lane 4) together with the not-size-fractionated chymotrypsin-modified PpPfk (lane 3) and the undigested PpPfk (lane 2). Precision Plus Protein Unstained Standard (BioRad) was used as molecular weight standard. Proteins were stained with Coomassie Blue R250.

Fig. 6. Immunoprecipitation of Pfks from cell-free extracts of P. pastoris wild type and the γ subunit-deficient strains.
Cell-free extracts from the P. pastoris wild type and the γ subunit-deficient strains were incubated with the anti-γ subunit antibody. The resulting immunocomplexes were precipitated with protein-A sepharose. Samples of purified PpPfk (lane 1, positive control), the cell-free extracts of the wild type (lane 2) and the γ subunit-deficient (lane 3) P. pastoris stains, the supernatants of both extracts after immunoprecipitation (lanes 4,5) and the respective protein A immunoprecipitates (lanes 6,7) were subjected to SDS-PAGE using a 10% polyacrylamide gel. Separated proteins were blotted and PpPfk subunits were detected using polyclonal antibodies against the α/β subunits and against the γ subunit.
Fig. 7. Reconstitution of PpPfk in cell-free extract of mixed subunit deletion mutants.
For in vitro reconstitution of PpPfk, two P. pastoris strains individually lacking one of the three subunit types were mixed equally prior cell disruption. (A) Cell-free extracts of the indicated individual and mixed P. pastoris strains were analyzed by Western blotting using polyclonal antibodies against α/β subunits and against the γ subunit of Pfk. (B) To demonstrate proper assembly of the PpPfk complex, cell-free extracts of all combinations of mutant strains were subjected to immunoprecipitation with the γ subunit-specific antibody. Pellets from protein-A-based immunoprecipitation were analyzed by Western blotting using polyclonal antibodies against the α/β subunits. (C) Specific PpPfk activity was monitored at 0.3 mM ATP. (D) The activation at 20 µM Fru 2,6-P_2 (given as fold activity over the activity without Fru 2,6-P_2) was measured at 0.3 mM ATP (black bars) and 5 mM ATP (white bars). In both experiments, Fru 6-P concentration was kept constant at a physiological level of 0.3 mM. Data are represented as mean ± S.D. of at least three independent experiments each performed in duplicate.
Table 1. Protein sequence analysis of the γ subunit of PpPfk.

Purified PpPfk from *P. pastoris* strain MH458 was subjected to SDS-PAGE. The γ subunit, co-purified with the α and β subunits, was excised and partially sequenced by Edman and MALDI-TOF PSD techniques (*). In-gel tryptic digestion and MALDI-TOF analysis were used to verify the integrity of the C-terminal part of the γ subunit (**).

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Table 2. Comparison of kinetic properties of purified Pfks from *P. pastoris* wild type and γ subunit-deficient strains.

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<th><em>PpPfk</em> γ-deficient</th>
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<td>$n_H^{\text{Fru}6-P}$</td>
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<td>+ 1 mM AMP</td>
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<td>$K_{0.5}^{\text{Fru}6-P}$ (mM)</td>
<td>0.17 ± 0.03</td>
<td>0.23 ± 0.03</td>
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<td>$n_H^{\text{Fru}6-P}$</td>
<td>1.30 ± 0.10</td>
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<td>+ 20 µM Fru 2,6-P$_2$</td>
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<td>$K_{0.5}^{\text{Fru}6-P}$ (mM)</td>
<td>0.28 ± 0.02</td>
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<td>1.20 ± 0.10</td>
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<td>half activation constants of allosteric effectors$^{(a)}$</td>
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<td>$K_A^{\text{Fru}2,6-P_2}$ (µM)</td>
<td>16.10 ± 1.10</td>
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<td>$n_H^{\text{Fru}2,6-P_2}$</td>
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<td>$K_A^{\text{AMP}}$ (mM)</td>
<td>0.12 ± 0.01</td>
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<td>$n_H^{\text{AMP}}$</td>
<td>1.60 ± 0.10</td>
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Residual Pfk activity <0.05 <0.05

Kinetic properties of the wild type and the γ subunit-deficient *PpPfk* were determined. All experiments were performed with purified enzymes (see suppl. Fig. S7). The constants refer to eq. 2 (see Experimental Procedures). Residual Pfk activity was defined as quotient of enzyme activity at 5.0 mM ATP and of enzyme activity at 0.3 mM ATP in absence of any activator. The values are means ± S.D. of two independent enzyme preparations.

(a) Measurement was carried out at 0.3 mM Fru 6-P.

(b) Data were obtained by measuring at 0.3 mM Fru 6-P without allosteric activator.
Table 3. Kinetic properties of purified Pfks from *P. pastoris* wild type and γ subunit-deficient strains calculated with a Monod-Wyman-Changeux model.

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<td>$V_{\text{MAX}}$ (mU/ml)</td>
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<td>$K_{S}^{\text{ATP}}$ (mM)</td>
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<td>$K_{T}^{\text{ATP}}$ (mM)</td>
<td>0.24 ± 0.12</td>
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<td>$K_{r}^{\text{Fru 2,6-P}}$ (μM)</td>
<td>12.70 ± 1.30</td>
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<td>$K_{r}^{\text{AMP}}$ (mM)</td>
<td>0.010 ± 0.005</td>
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The constants refer to eq. 1 (see *Experimental Procedures*). $K_{S}^{\text{ATP}}$ was determined from kinetic data measured at 6 mM Fru 6-P. All other data were obtained by measuring at 0.3 mM Fru 6-P and 3 mM ATP. Kinetic constants were calculated from data shown in suppl. Fig. S8. Note that $V_{\text{MAX}}$ values refer to different enzyme preparations why they can not be compared with each other.
Figure 1

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Figure 2

A

Cell density (OD₅₈₀nm)

0  5  10  15  20  25

Time (h)

B

Pfk activity (U/mg)

0  0.2  0.4  0.6  0.8  1.0

Time (h)
Figure 3

γ subunit-deficient strain
wild type strain

γ subunit-deficient strain
wild type strain

after 1 min

after 2 min
Figure 4

A

B

C

D

AMP (mM)

Fru 2,6-P2 (µM)

ATP (mM)

ATP (mM)

V_{AMP/V_0}

V_{Fru\,2,6-P_2/V_0}

V_{AMP/V_0}

V_{Fru\,2,6-P_2/V_0}

0.001 0.01 0.1 1

0.001 0.01 0.1 1

0 1 2 3 4 5

0 100 200 300 400

20 40 60 80 100 120 140 160

20 40 60 80 100 120 140 160

20 40 60 80 100 120 140 160

20 40 60 80 100 120 140 160

20 40 60 80 100 120 140 160

20 40 60 80 100 120 140 160
Figure 5

![SDS-PAGE gel showing molecular weight markers and protein bands](image)

- **Marker**
- **PpPfk**
- **Chymotrypsin-modified PpPfk**
- **PpPfk after HPLC gel filtration**

Molecular weights (kDa) indicated:
- 100 kDa
- 75 kDa
- 50 kDa
- 37 kDa
- 25 kDa

Protein bands labeled:
- α
- β
- γ
- γ'
Figure 6

![Image of the figure]

- IgG (heavy chain)
- Anti- subunit for precipitation
Figure 7

Extract(s) mixed and applied in the experiment

|                | + | - | - | - | - | - | wild type strain | + | - | - | - | - | - | α subunit-deficient strain | - | + | - | - | + | + |
|----------------|---|---|---|---|---|---|------------------|---|---|---|---|---|---| β subunit-deficient strain | - | - | + | - | + | + |
|                | - | + | - | + | - | - | γ subunit-deficient strain | - | - | + | + | + | + |                       |   |   |   |   |   |   |

A

B

C

D

Extract(s) mixed and applied in the experiment

|                | + | - | - | - | - | - | wild type strain | + | - | - | - | - | - | α subunit-deficient strain | - | - | + | + | - | - |
|----------------|---|---|---|---|---|---|------------------|---|---|---|---|---|---| β subunit-deficient strain | - | - | + | - | + | + |
|                | - | - | + | - | + | - | γ subunit-deficient strain | - | + | + | + | + | + |                       |   |   |   |   |   |   |
A novel form of 6-phosphofructokinase: Identification and functional relevance of a third type of subunit in Pichia pastoris
Katrin Tanneberger, Jürgen Kirchberger, Jörg Bär, Wolfgang Schellenberger, Sven Rothemund, Manja Kamprad, Henning Otto, Torsten Schoneberg and Anke Edelmann

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