

# Interaction of murine monoclonal subunit-specific antibodies with phosphofructokinase-1 from *Saccharomyces cerevisiae*\*\*

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**Abstract** Murine monoclonal subunit-specific antibodies against heterooctameric phosphofructokinase-1 from *Saccharomyces cerevisiae* exhibiting no cross-reactivity were purified and characterized regarding complex formation with the native enzyme, immunological reactivity to the SDS-denatured subunits originating from native and proteolytically truncated phosphofructokinase, and protection against proteolytic degradation. Strong complex formation was found with one  $\alpha$ -specific antibody. Western blotting employing different enzyme forms allowed to localize epitope sites of the  $\alpha$ -polypeptide chain. Monoclonal antibodies protect phosphofructokinase-1 against chymotryptic degradation.

**Key words:** Phosphofructokinase (yeast); Monoclonal antibody; Subunit-specificity; Proteolysis

## 1. Introduction

The octameric phosphofructokinase-1 from *Saccharomyces cerevisiae* (Pfk-1) is composed of two types of subunits ( $\alpha$  and  $\beta$ ) in equal ratio which are encoded by the genes *PFK1* and *PFK2*, respectively [1,2]. Their deduced amino acid sequences show about 20% of homology in the N- and C-terminal regions and more than 50% of homology in the middle part of both polypeptide chains [3].

Therefore, polyclonal antibodies raised against the native enzyme should recognize both types of subunits. In fact, in earlier studies applying two-dimensional immunoelectrophoresis we could demonstrate that polyclonal antibodies are capable of distinguishing between  $\alpha$  and  $\beta$  as indicated by crossing precipitation lines [4]. This finding was the first evidence for the existence of different types of subunits in yeast Pfk-1. In addition, the fractionation of polyclonal antibodies against Pfk-1 by affinity chromatography on the immobilized separated subunits resulted in three fractions: antibodies which recognize both types of subunits, antibodies which are exclusively directed against the  $\alpha$ -polypeptide chain, and antibodies which specifically bind to the  $\beta$ -subunit [5].

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\*\*Dedicated to Prof. Dr. E. Hofmann (Leipzig) on the occasion of his 65th birthday.

**Abbreviations:** Pfk-1, phosphofructokinase-1 (EC 2.7.1.11); mAb, monoclonal antibody; HAT, hypoxanthin-aminopterin-thymidin; DMEM, Dulbecco's modified Eagle medium; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulphate; aa, amino acid of unknown structure.

All these antibodies were found to inhibit the catalytic activity of the enzyme, however, to different extents.

In this paper, the production of murine monoclonal antibodies distinctively recognizing each type of subunits of Pfk-1 without showing any cross-reactivity is described. The antibodies were characterized regarding complex formation with the native enzyme, immunological reactivity to the denatured subunits originating from native and proteolytically truncated yeast Pfk-1, and protecting effect against proteolytic degradation.

## 2. Experimental

### 2.1. Reagents and enzymes

The enzyme was purified according to Hofmann and Kopperschläger [6], with minor modifications. Ammonium sulphate of the first fractionated precipitation of the enzyme was replaced by poly(ethylene glycol) 6000 (4% and 12%, respectively). As source the recombinant strain PS1 of *Saccharomyces cerevisiae* (*MATa ura3-52 leu2-3,112 his4-519 pep4-3 gal2 cir<sup>+</sup>*) carrying the *PFK1* and *PFK2* gene in a multicopy vector was used. PS1 is the *cir<sup>+</sup>*-form of the strain PS2 described by Seeboth et al. [7].

The biochemicals for the Pfk-1 assay were from Boehringer (Mannheim, Germany). All other analytical reagent grade chemicals used were from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany).

### 2.2. Methods

**2.2.1. Production of monoclonal antibodies.** Female BALB/c mice (10 weeks old) were immunized by intraperitoneal injection of 100  $\mu$ g purified Pfk-1 in complete Freund's adjuvant. 12 weeks after the first injection, the animals were boosted on three consecutive days with 50  $\mu$ g phosphofructokinase dissolved in 50 mM sodium phosphate buffer, pH 7.0, 150 mM sodium chloride. One day after the last injection, spleen cells ( $3 \times 10^7$ ) were fused with mouse myeloma cells (P3-X63-Ag8.653) using polyethylene glycol 1500 according to Köhler and Milstein [8].

Hybridoma were grown in DMEM (Gibco, Berlin, Germany) supplemented with HAT in the presence of 10% fetal calf serum. Positive clones were recognized by screening the supernatants in an ELISA using native Pfk-1 for coating 96-well microtiter plates (Nunc, Wiesbaden, Germany). The class and subclass specificity was determined by using a commercial kit (Virotec, Rüsselsheim, Germany). The selected clones were recloned by the limiting dilution method. Subunit-specificity was assessed from the recognition of the  $\alpha$ - and  $\beta$ -chain electroblotted from SDS-polyacrylamide gels (Western blotting).

**2.2.2. Purification of monoclonal antibodies.** Subunit-specific clones were grown in DMEM containing 10% fetal calf serum at 37°C for several days in culture flasks. The culture supernatant was collected and the protein was precipitated with ammonium sulphate (100% saturation) at 4°C, followed by dialysis against 20 mM sodium phosphate buffer, pH 7.0. The dialyzed protein was applied onto a protein A-sepharose column equilibrated with the dialysis buffer. The protein was eluted with 100 mM citrate buffer, pH 3.0. For immediate neutralization, 1 M Tris-HCl, pH 7.0, was used. The antibodies were concentrated again by ammonium sulphate precipitation, re-dissolved in 50 mM

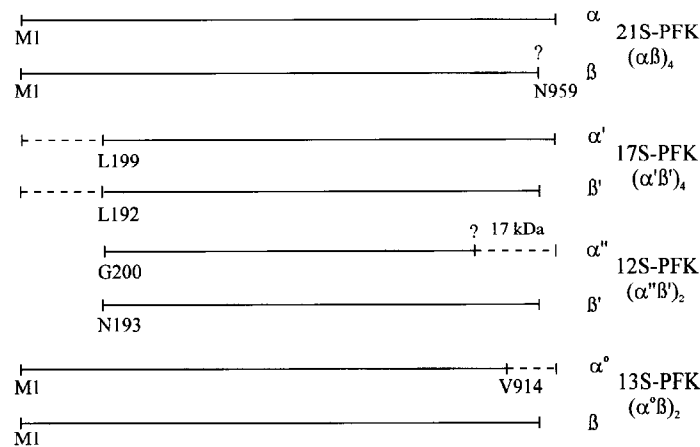


Fig. 1. Truncated polypeptide chains of phosphofructokinase from *S. cerevisiae* (according to [9]).

sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, precipitated at ammonium sulphate saturation and stored at 0°C.

**2.2.3. Polyacrylamide gel electrophoresis and Western blotting.** SDS-polyacrylamide gel electrophoresis was performed in a Bio-Rad Mini-Protean II Dual slab cell apparatus as described elsewhere [9] using 7.5% acrylamide running gels. The subunit specificity of the monoclonal antibodies was checked by immunoblotting according to Towbin et al. [10]. SDS-denatured purified Pfk-1 (5–10 µg per lane) was subjected to SDS-polyacrylamide gel electrophoresis, followed by electrotransfer of the separated subunits to a nitrocellulose membrane NC 45 (Serva, Heidelberg, Germany). The membrane was washed, temporarily stained with Ponceau S (0.2 g dye per 100 ml of 12% trichloroacetic acid), and then treated with the respective monoclonal antibody as described by Clifton and Fraenkel [11]. Detection was carried out with sheep anti-mouse immunoglobulin/peroxidase conjugate (Boehringer, Mannheim, Germany).

**2.2.4. Assay of phosphofructokinase activity.** The catalytic activity of Pfk-1 was measured spectrophotometrically at 25°C using a coupled enzyme assay containing 100 mM imidazole-HCl, pH 7.2, 3 mM Fru 6-P, 0.6 mM ATP, 1 mM AMP, 5 mM MgSO<sub>4</sub>, 5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2 mM NADH, aldolase (1 U/ml), glycerolphosphate dehydrogenase (1.5 U/ml), and triosephosphate isomerase (5 U/ml). The reaction was started by the addition of 20–50 µl Pfk-1 appropriately diluted with 50 mM sodium phosphate buffer, pH 7.0.

**2.2.5. HPLC size-exclusion chromatography.** Isocratic size-exclusion chromatography was performed by FPLC on a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden) or on a Biosil SEC-400 column (Bio-Rad) coupled to a Pharmacia HPLC-chromatograph. The column was equilibrated and run with 50 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.0, at a flow-rate of 1 ml/min. In some experiments sodium chloride was omitted. The absorbance of the efflu-

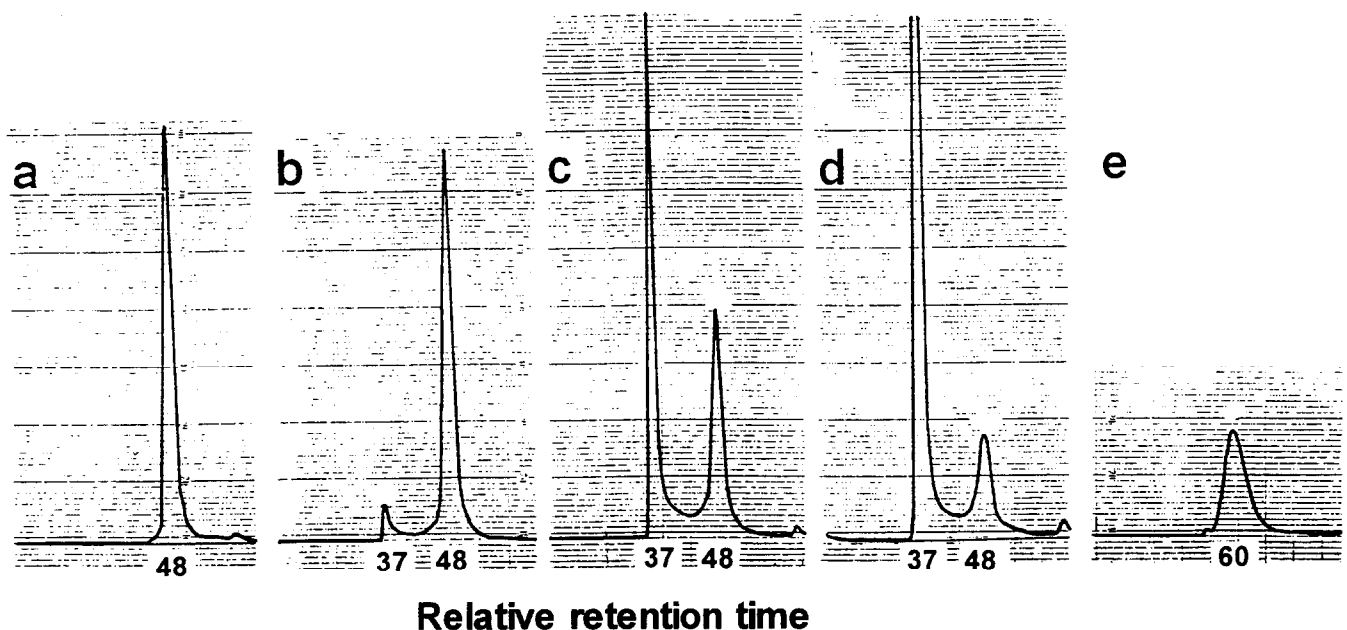


Fig. 2. Detection of immunocomplexes consisting of  $\alpha$ -subunit-specific monoclonal antibody and octameric phosphofructokinase from *S. cerevisiae*. A mixture consisting of the mAb  $\alpha$ -F3 and native Pfk-1 was fractionated on a Superose 12 HR 10/30 HPLC column in 50 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl. The protein amounts (antibody/phosphofructokinase) applied onto the column in 100 µl buffer were (a) 50/0 µg; (b) 50/10 µg; (c) 50/50 µg; (d) 50/100 µg; and (e) 0/100 µg, respectively. The relative retention time (quotient of the elution time of the corresponding protein and the elution time of dinitrophenyl alanine  $\times$  100) of 37, 48, and 60 corresponds to a molecular mass of higher than 1,300 kDa, 708 kDa, and 141 kDa, respectively, as estimated from a calibration curve.

ent was continuously monitored at either 226 nm or 280 nm. Both columns were calibrated with Sigma standard proteins.

**2.2.6. Exposure of Pfk-1 to chymotrypsin.** Pfk-1 dissolved in 50 mM sodium phosphate buffer, pH 7.0, was mixed with the respective mAb dissolved in the same buffer to give final concentrations of 5 pM, respectively, related to the half molecule of Pfk-1 (2 mg Pfk-1/ml; 0.75 mg mAb/ml) and was incubated for 15 min at 23–25°C. Chymotrypsin was added (final concentration 250 µg/ml) and samples were withdrawn periodically for assaying Pfk-1 activity. Proteolysis was stopped after 4 h by the addition of PMSF (final concentration 1 mM).

### 3. Results

#### 3.1. Characterization of mAbs by Western blotting with different forms of phosphofructokinase

From hybridoma cells of mice previously immunized with native Pfk-1 four  $\alpha$ -specific and five  $\beta$ -specific mAbs were selected and characterized as indicated in Table 1. With the exception of mAb  $\beta$ -4B3 which was found to be IgG2a all other mAbs belong to the IgG1 subclass.

In order to localize the interacting epitopes of Pfk-1, the proteolytically modified forms of the enzyme depicted in Fig. 1 were employed in Western blot analysis. As seen in Table 1, the complete set of  $\beta$ -specific mAbs reacted with all the fragments of the  $\beta$ -chain. Similarly, mAb  $\alpha$ -3E10 gave a signal with the native as well as with all forms of the truncated  $\alpha$ -subunit. The mAb  $\alpha$ -3G7 recognized native 21 S-Pfk-1, 17 S-Pfk-1 and 13 S-Pfk-1. In contrast, mAb  $\alpha$ -F3 was reactive with native 21 S- and 13 S-Pfk-1 only, whereas mAb  $\alpha$ -3B3 recognized the 21 S- and the 17 S-enzyme.

#### 3.2. Size exclusion chromatography of mAb-Pfk-1 complexes

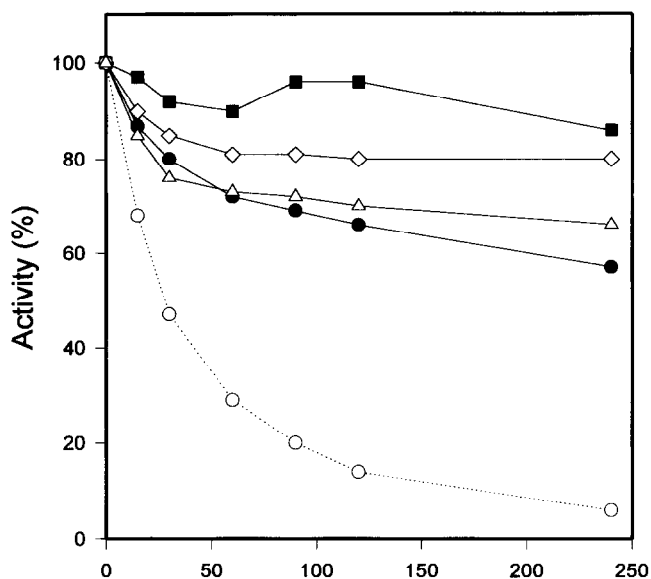
For studying the interaction of subunit-specific antibodies with the oligomeric, non-denatured Pfk-1 (21 S), antigen and mAb were incubated at different molar ratios. The mixtures were fractionated on a Superose 12HR 10/30 FPLC column or, alternatively, on a Biosil SEC 400 column.

A stable immunocomplex consisting of the subunit-specific antibody and octameric Pfk-1 was identified with mAb  $\alpha$ -F3 only (Fig. 2). Due to the molecular weight to be expected of approximately 1,000 kDa the complex appeared in the void volume of the column. Complex formation turned out to occur within seconds as seen from gel filtration experiments where both proteins were mixed immediately before application onto the column. A prolongation of the incubation time up to 3 h did not influence the extent of complex formation.

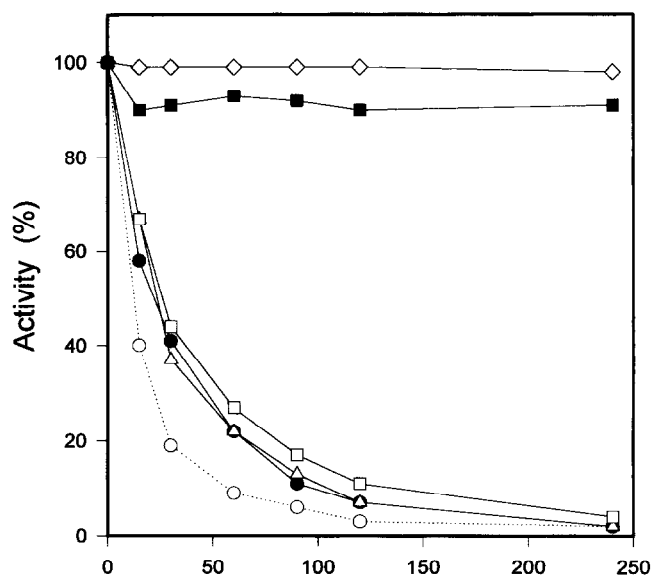
Table 1

Reactivity of subunit-specific monoclonal antibodies to truncated forms of yeast phosphofructokinase-1 as detected by Western-blot analysis

Monoclonal antibody	21 S-Pfk-1	17 S-Pfk-1	13 S-Pfk-1	12 S-Pfk-1
$\alpha$ -F3	+	–	+	–
$\alpha$ -3G7	+	+	+	–
$\alpha$ -3B3	+	+	–	–
$\alpha$ -3E10	+	+	+	+
$\beta$ -3G2	+	+	+	+
$\beta$ -3D4	+	+	+	+
$\beta$ -1B11	+	+	+	+
$\beta$ -4B3	+	+	+	+
$\beta$ -3B5	+	+	+	+



A Time (min) after chymotrypsin addition



B Time (min) after chymotrypsin addition

Fig. 3. Time course of the catalytic activity of phosphofructokinase from *S. cerevisiae* during proteolysis in the presence of monoclonal antibodies. Pfk-1 (2 mg/ml) dissolved in 50 mM sodium phosphate buffer, pH 7.0, was incubated at 23–25°C for 15 min with equimolar amounts of mAbs before chymotrypsin was added in a volume of 20 µl (final concentration 250 µg/ml). 20 µl samples were withdrawn periodically and assayed for Pfk-1 activity. (A) ○, control without antibody; ■, mAb  $\alpha$ -3B3; ◇, mAb  $\alpha$ -E10; △, mAb  $\alpha$ -3G7; ●, mAb  $\alpha$ -F3. (B) ○, control without antibody; ◇, mAb  $\beta$ -3B5; ■, mAb  $\beta$ -3D4; □, mAb  $\beta$ -1B11; ●, mAb  $\beta$ -4B3.

Immunocomplexes of Pfk-1 with  $\beta$ -3D4 and  $\beta$ -1B11 were also detected using the antibody in excess.

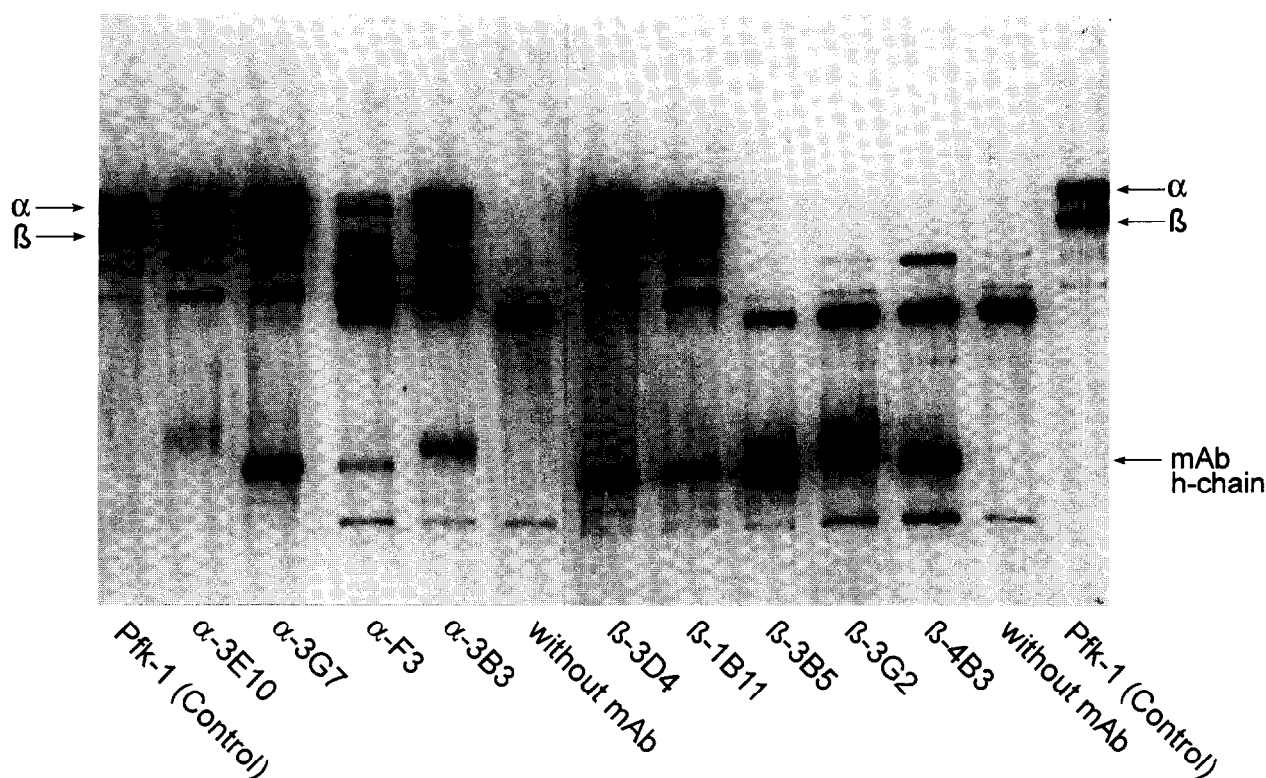


Fig. 4. SDS-polyacrylamide gel electrophoresis of phosphofructokinase from *S. cerevisiae* after chymotryptic digestion. The mixtures of Pfk-1, mAb and chymotrypsin, characterized in Fig. 3, were incubated for 4 h at 23–25°C. Proteolytic activity was stopped by the addition of PMSF (final concentration 1 mM). The samples were heat-denatured in 3 vols. SDS-cocktail (0.06 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.5 M 2-mercaptoethanol, 0.0001% Bromophenol blue). 10 µg protein/lane were subjected to electrophoresis (45 min at 200 V).

### 3.3. Protective effect of mAbs against proteolytic degradation of Pfk-1

The influence of the mAbs on the chymotryptic degradation of Pfk-1 was studied by analyzing both catalytic activity and peptide pattern following proteolytic digestion. A possible inhibition of chymotryptic activity by mouse immunoglobulin was ruled out by a control experiment.

As depicted in Fig. 3A, the activity of Pfk-1 decreased rapidly within 4 h reaching 10% in the absence of any mAb due to the chymotryptic action. After addition of the mAb  $\alpha$ -3B3,  $\alpha$ -3E10,  $\alpha$ -3G7 or  $\alpha$ -F3, respectively, the enzyme activity was more or less preserved. Maximum protection with remaining activity of 80–90% was found in the presence of mAb  $\alpha$ -3B3 and  $\alpha$ -3E10 whereas  $\alpha$ -3G7 and  $\alpha$ -F3 were found to act less protective.

Among the  $\beta$ -specific antibodies,  $\beta$ -1B11 and  $\beta$ -3D4 almost completely protected the enzyme against proteolytic inactivation (Fig. 3B). The presence of mAbs  $\beta$ -3B5,  $\beta$ -3G2, or  $\beta$ -4B3 did not influence chymotryptic inactivation of Pfk-1.

In order to compare the changes in enzyme activity with alterations in the structure of Pfk-1, the subunit pattern was analyzed following chymotrypsin treatment by SDS-polyacrylamide gel electrophoresis (Fig. 4).

No significant proteolytic degradation of the  $\alpha$ - or  $\beta$ -subunit of Pfk-1 was observed in the presence of the mAbs  $\alpha$ -3E10,  $\alpha$ -3G7,  $\beta$ -1B11, or  $\beta$ -3D4, respectively. In contrast, the control experiment lacking the mAb revealed a complete degradation of the two types of subunits to low molecular weight fragments.

The effect of mAbs  $\alpha$ -3B3 and  $\alpha$ -F3 is of particular interest:

a limited degradation of both subunits was observed, leading to a pair of peptides with molecular weights of 87 and 76 kDa, respectively. In the presence of mAb  $\alpha$ -3B3, the  $\alpha$ -polypeptide chain seems to be more protected than in presence of  $\alpha$ -F3. Western blotting of the peptides with monoclonal antibodies revealed the 87 kDa-band originates from the  $\alpha$ - and the 76 kDa-peptide from the  $\beta$ -subunit, respectively.

### 4. Discussion

Despite significant similarities in the primary structure of the  $\alpha$ - and  $\beta$ -chain of yeast Pfk-1, monoclonal antibodies were generated and purified exhibiting subunit specificity. The interaction of  $\alpha$ - and  $\beta$ -specific mAbs with SDS-denatured Pfk-1 as studied by Western blotting resulted in responses depending on the type of the truncated subunit applied and suggesting the following classification: class I, mAbs which bind either to the N- or C-terminal part of the polypeptide chains, and class II, mAbs recognizing epitopes that belong to the inner part of the subunits.

Those mAbs which recognize peripheral epitopes ( $\alpha$ -F3,  $\alpha$ -3G7 and  $\alpha$ -3B3) are exclusively  $\alpha$ -specific. The mAb  $\alpha$ -F3 is able to detect 21 S- and 13 S-Pfk-1 but not the 17 S- and 12 S-form. In the first two enzyme species, the complete N-terminal part of the  $\alpha$ -polypeptide chain is preserved, whereas the latter two are lacking it (Fig. 1). This finding suggests a localization of the epitope for mAb  $\alpha$ -F3 within the N-terminal 200 amino acids of the  $\alpha$ -polypeptide chain.

The mAb  $\alpha$ -3B3 recognized both native Pfk-1 and the 17 S-enzyme but did not interact with the 13 S- and 12 S-form. Because the  $\alpha$ -chain is degraded in the latter two enzyme species at the C-terminus, it becomes likely that the epitope for  $\alpha$ -3B3 belongs to the C-terminal part of the subunit.

Similarly, a C-terminal epitope was recognized by the mAb  $\alpha$ -3G7. This antibody showed interaction with all forms of Pfk-1 with the exception of the 12 S-species. Since the cleavage site of the  $\alpha$ -chain to form 12 S-Pfk-1 was found in the vicinity of aa<sup>830</sup> as calculated from the released peptide [9], and the cleavage site in the  $\alpha$ -polypeptide chain to form 13 S-Pfk-1 is Val<sup>914</sup> (see Fig. 1) the conclusion can be drawn that the epitope for the mAb  $\alpha$ -3G7 is localized between aa<sup>830</sup> and Lys<sup>913</sup> of the  $\alpha$ -polypeptide chain. The class I antibodies may be taken as indication for the unique exposure of the  $\alpha$ -ends of the polypeptide chain.

In contrast, the  $\beta$ -specific mAbs investigated were reactive to all forms of truncated Pfk-1. This finding indicates that their epitopes are localized in the middle part of the  $\beta$ -polypeptide chain which is unaffected in the course of limited proteolysis. Pfk-1 is rapidly degraded by the attack of chymotrypsin and other proteases which is followed by inactivation and by the release of peptides. Substrates and effectors of the enzyme turned out to be capable of maintaining enzyme activity due to a limitation of proteolysis [9].

In this study, four  $\alpha$ -specific mAbs ( $\alpha$ -3B3,  $\alpha$ -3E10,  $\alpha$ -3G7 and  $\alpha$ -F3) were found to preserve enzyme activity from chymotryptic inactivation. Therefore, these antibodies must complex the enzyme under the conditions of proteolysis. However, in most cases (with the exception of  $\alpha$ -F3) low complex stability allows dissociation of the immunocomplex during HPLC gel filtration.

From the set of  $\beta$ -specific mAbs only  $\beta$ -1B11 and  $\beta$ -3D4 are capable to protect the enzyme from proteolytic inactivation although complex formation could be detected by HPLC gel filtration with  $\beta$ -3D4 only.

The degree of chymotryptic digestion was found to coincide qualitatively with the extent of inactivation. Independent of the type of antibody essentially no subunit degradation was detected with the majority of antibodies completely preserving enzyme activity with the exception of  $\alpha$ -3B3 and  $\alpha$ -F3. In the presence of  $\alpha$ -3B3, the degradation of the  $\alpha$ -subunit was substantially retarded finally leading to a truncated polypeptide of

87 kDa. It should be stressed, that the accompanying limited breakdown of the  $\beta$ -subunit to a 76 kDa fragment was unexpected. Obviously, under these conditions an enzyme species is formed which is similar or identical to the truncated but catalytically active 17 S-Pfk-1 [9]. In a resembling way, the chymotryptic proteolysis of Pfk-1 occurring in the presence of  $\alpha$ -F3 generates a pair of truncated polypeptides with comparable molecular weights. However, the protection of the native  $\alpha$ -subunit is less pronounced than in the case of  $\alpha$ -3B3.

The results of this study let us assume that the Pfk-1 antibody interaction is based on sequence rather than on conformational determinants of the enzyme. The  $\beta$ -type antibodies react with all forms of Pfk-1 independent of the oligomeric state of the enzyme. Also,  $\alpha$ -type antibodies recognize both the octameric and the tetrameric enzyme except for mAb  $\alpha$ -3B3 which is reactive with the octameric form of Pfk-1 only. This behaviour could also be explained as a structure-specific interaction with epitopes being present exclusively in octameric Pfk-1.

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