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Purification of the Ca^{2+}-binding protein S100A1 from myocardium and recombinant *Escherichia coli*

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Abstract

S100A1 is a new regulatory protein of myocardial contractility that is differentially expressed in early and late stages of myocardial hypertrophy. In order to further investigate the multiple functions of S100A1 in various assay systems we developed a new strategy for isolating biologically active S100A1 protein. After EDTA extraction of myocardium or recombinant bacteria, S100A1 was purified by Octyl-Sepharose hydrophobic interaction chromatography and HiTrapQ anion-exchange chromatography yielding 1.4–2.0 mg/100 g wet tissue and 0.7–1.0 mg/100 ml bacterial culture. Native porcine as well as human recombinant S100A1 revealed biological activity in physiological and biochemical assays.

Keywords: Purification; *Escherichia coli*; S100A1; Calcium-binding proteins

1. Introduction

S100 proteins belong to the large family of calcium binding proteins with EF-hand type Ca^{2+}-binding motives (for review see Ref. [1]). As initially detected by Moore [2] they were named S100 according to their solubility in 100% ammonium sulfate solution. In contrast to calmodulin, S100 proteins reveal a tissue specific expression pattern thus translating the ubiquitous Ca^{2+} signal into a tissue specific response. So far 16 S100 proteins have been discovered being involved in the regulation of cell differentiation, cell cycling, signal transduction and cytoskeletal architecture [3].

Among these proteins S100A1 displays a specific expression pattern in slow skeletal and cardiac muscle [4]. Since S100A1 is involved in the activation of the sarcoplasmatic calcium release [5,6] and the regulation of intermediate filament polymerisation [7,8] the finding of significantly downregulated S100A1 protein levels in human end stage heart failure [9] and upregulated levels in compensated hypertrophy [10] might be of pathophysiological relevance in explaining the molecular basis of impaired contractile performance in human cardiomyopathy.

To further elucidate the multiple functions of S100A1 in biochemical and biophysical experiments larger amounts of highly purified and biologically active protein are needed. Previous purification protocols either employed several chromatographic and time consuming dialysis steps [11] or include a

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reversed-phase chromatography step [12] thus leading to a considerable loss of protein and denatured biomolecules respectively. We therefore developed a new purification method for native and recombinant S100A1 protein that yields high amounts of biologically active protein within 24 h.

2. Experimental

2.1. Reagents and materials

All chemicals were obtained from Merck (Darmstadt, Germany) if not indicated otherwise. Porcine tissue was obtained from the local slaughter-house.

2.2. Tissue extraction and recombinant proteins

Myocardial samples were homogenized in five volumes (v/w) of ice cold extraction buffer [25 m*M* Tris–HCl, 50 m*M* KCl, 5 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma, Munich, Germany), pH 7.5] and centrifuged (10 000 *g*, 15 min, 4°C). Supernatants were adjusted to 50% ammonium sulfate saturation, equilibrated for 15 min at room temperature, and centrifuged (10 000 *g*, 15 min, 4°C). Recombinant protein was expressed in *E. coli* of the BL21 (DE3) Lys S-strain using a pGEMEX T7 expression vector (Promega, Madison, WI, USA) containing the human cDNA of S100A1 [13]. LB medium (Gibco/Life Technologies, Gaithersburg, MD, USA) containing 100 mg/l ampicillin and 30 mg/l chloramphenicol was inoculated with a 5 ml overnight culture of an *E. coli* clone expressing the human S100A1 protein. At an optical density at 600 nm ($O.D._{600}$) of 0.5 the expression of recombinant protein was induced with 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG, Sigma). After further incubation for 4 h bacteria were spun down (10 000 *g*, 15 min, 4°C) and resuspended in 10 ml of lysis buffer (20 m*M* Tris–HCl, 50 m*M* KCl, 5 m*M* EDTA, 1 m*M* PMSF, pH 7.5). Cell lysis was performed by two cycles of freezing and thawing followed by sonification for 1 min on ice. After centrifugation (10 000 *g*, 15 min, 4°C) the resulting supernatants were adjusted to 50% ammonium sulfate and processed as described for myocardial tissues.

2.3. Hydrophobic interaction chromatography (HIC)

All chromatography steps were run on an Äkta Purifier high-performance liquid chromatography (HPLC) system (Pharmacia, Uppsala, Sweden) using the Unicorn 2.20 software (Pharmacia). After saturation with ammonium sulfate and centrifugation (10 000 *g*, 15 min, 4°C) the resulting supernatants were injected onto different HIC columns using the HiTrap HIC Test Kit (Pharmacia) equilibrated with loading buffer (25 m*M* Tris–HCl, 2 m*M* $CaCl_2$, pH 7.5). Elution of Ca^{2+}-binding proteins was achieved by a step-gradient with elution buffer [25 m*M* Tris–HCl, pH 9.5, 5 m*M* ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 9.5; 1 ml/min]. UV monitoring was performed at 280 nm. Eluting Ca^{2+}-binding proteins were quantified by peak integration and further analyzed by reversed-phase HPLC according to Remppis et al. [9]. The purification procedure was finally upgraded for Octyl-Sepharose (XK26 column holder, Pharmacia, 47.8 ml, 10×2.6 cm, constant flow 5 ml/min).

2.4. Anion-exchange chromatography (AIEX)

Ca^{2+}-binding proteins eluting with the EGTA step-gradient were collected in aliquots of 5 ml and then loaded onto a HiTrapQ anion exchanger (Pharmacia; 5 ml, 2.5×1.6 cm) equilibrated with a low-salt buffer (25 m*M* Tris–HCl, pH 7.5). Proteins were then eluted by a linear gradient from 0% to 40% high-salt buffer (25 m*M* Tris–HCl, 1 *M* NaCl, pH 7.5) within 40 min (flow-rate 5 ml/min). UV monitoring was performed at 220 nm.

2.5. Analytical methods

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), samples were diluted in sample buffer (0.5 *M* Tris–HCl, 5% SDS, 10% glycerol, 0.05% bromphenolblue, 5% β-mercaptoethanol, pH 6.8) and boiled for 3 min. Aliquots (10 μl) were separated on SDS–polyacrylamide gels (15% T, 3% C) using the Tris–tricine buffer system [14]. The proteins were transferred to polyvinylidene difluoride (PVDF) sheets (Bio-Rad, Hercules, CA,

USA) with a semi-dry transfer system [15]. The immunodetection of proteins was performed using the Western Light Plus kit (Tropix, Bedford, MA, USA) employing the CSPD chemiluminescence system according to the manufacturer's guidelines. Primary antibodies were diluted 1:10 000 in blocking buffer, while the working dilution of the biotin-labeled secondary antibody and the streptavidin–alkaline phosphatase conjugate was 1:20 000.

Two-dimensional electrophoresis [16] was carried out using the Immobiline DryStrip Kit and ExcelGel SDS–PAGE on a Multiphor II electrophoresis system (all Pharmacia) according to the manufacturer's guidelines. Silver staining of polyacrylamide gels was performed according to Oakley et al. [17].

The protein content was determined using the DC Protein Assay (Bio-Rad) according to a modification of the method described by Lowry et al. [18].

Inhibition of phosphoglucomutase by S100A1 was assayed at concentrations of 1 μ*M* S100A1 and 1 m*M* $CaCl_2$ on a Uvikon 930 photometer (Kontron, Eching, Germany) using the procedure described by the manufacturer (Boehringer Mannheim, Germany) according to Landar et al. [19].

3. Results and discussion

The binding of Ca^{2+} to S100 proteins leads to a coordinated presentation of hydrophobic patches at the surface of these molecules forming a hydrophobic site for the interaction with their target proteins [20]. These Ca^{2+} dependent conformational changes may be exploited for the purification of EF-hand Ca^{2+}-binding proteins by the use of HIC.

After EDTA extraction of Ca^{2+}-binding proteins the resulting supernatants were fractionated by ammonium sulfate. This leads to a salting-out of proteins with a M_r greater than approximately 50 000, while smaller Ca^{2+}-binding proteins remain in solution showing a strong hydrophobic interaction with matrices like Octyl-, Butyl- and Phenyl-Sepharose. After injecting the supernatants onto HIC columns we used a Ca^{2+} washing buffer to eliminate an unspecific hydrophobic binding of proteins mediated by ammonium sulfate while the Ca^{2+} related binding of S100 proteins is stabilized. Since gentle binding and elution conditions are prerequisites for a high yield of highly purified and biologically active proteins we tested Octyl-, Butyl- and Phenyl-Sepharose matrices for their different binding characteris-

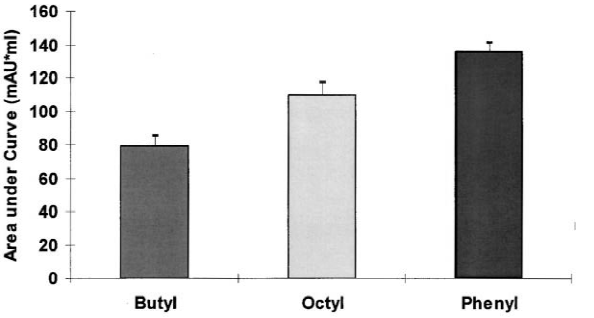


Fig. 1. Ca^{2+}-binding protein pools eluted with 5 m*M* EGTA from Butyl-, Octyl- and Phenyl-Sepharose columns quantified by peak integration at 280 nm.

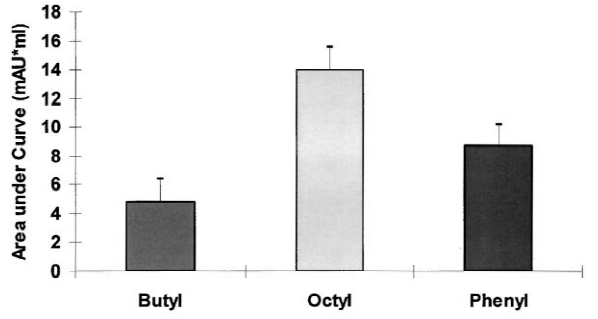


Fig. 2. S100A1 protein fraction from Ca^{2+}-binding protein pools eluted from Butyl-, Octyl- and Phenyl-Sepharose as analyzed by reversed-phase chromatography and quantified by peak integration at 280 nm.

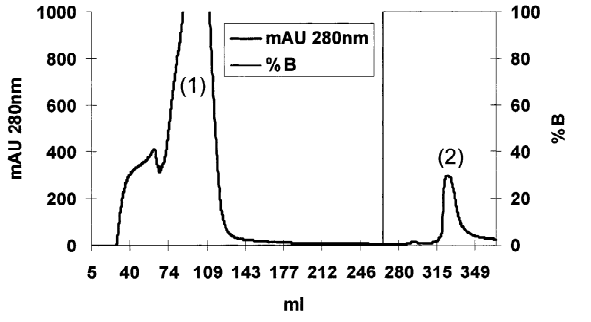


Fig. 3. Octyl-Sepharose hydrophobic interaction chromatography (HIC) of porcine ammonium sulfate supernatants: fall through (peak 1), EGTA eluate of Ca^{2+}-binding proteins (peak 2).

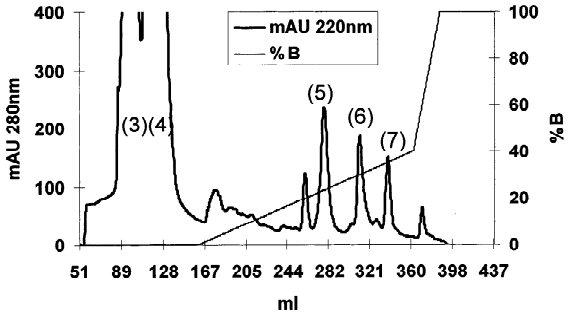


Fig. 4. Q-Sepharose anion-exchange chromatography (AIEX) of porcine ammonium sulfate supernatants: fractionation of the EGTA eluates from Octyl-Sepharose (Fig. 1). S100A1 elutes with peaks 5 and 6.

tics. As it is depicted in Fig. 1 recovery of Ca^{2+} dependently bound proteins was highest from Phenyl-Sepharose. Further evaluation of these EGTA peaks by reversed-phase however showed the highest S100A1 protein recovery from Octyl-Sepharose (Fig. 2). The EGTA elution peaks from Octyl-Sepharose (Fig. 3; peak 2) were directly loaded onto a HiTrapQ anion-exchange column, without any intermediate dialysis step. With a linear gradient from 0 to 400 m*M* NaCl S100A1 eluted at a concentration of 300 m*M* (Fig. 4, peaks 5 and 6). Fig. 5 shows the SDS–PAGE (a) and Western blotting analyses (b) of the resulting protein peaks from HIC and AIEX. Lane 1 in Fig. 5 shows the wash-out of proteins by the Ca^{2+} washing buffer that bound to the Octyl-Sepharose matrix in an ammonium sulfate dependent way while the EGTA peak (lane 2) mainly contains small Ca^{2+}-binding proteins as reported recently [9]. S100A1 bound quantitatively to Octyl-Sepharose as lane 1 does not show any S100A1 immunoreactivity (Fig. 5b). Peak 6 contained monomeric S100A1 as well as larger amounts of homodimeric S100A1 (Fig. 5a and b). Since the applied SDS–PAGE system does not show a resolution sufficient to differentiate between several proteins with a M_r near 10 000 (compare lanes 1 and 2 in Fig. 5a) we additionally confirmed the purity of our S100A1 preparations by two-dimensional PAGE. While Fig. 6a depicts a two-dimensional run of a whole EDTA extraction of

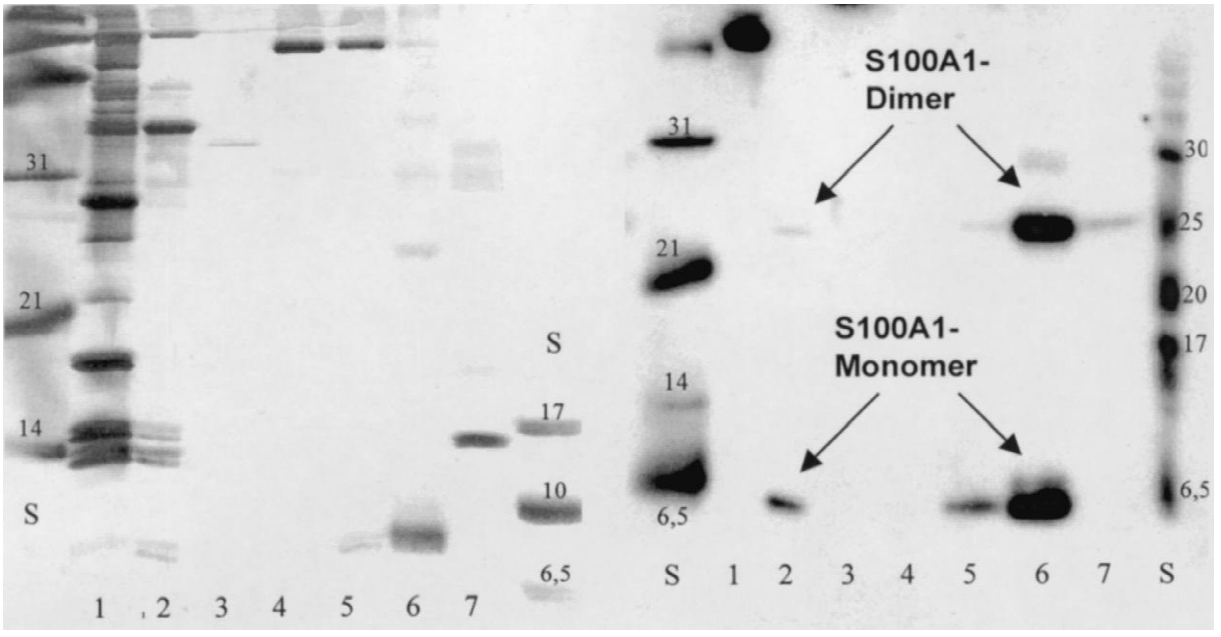
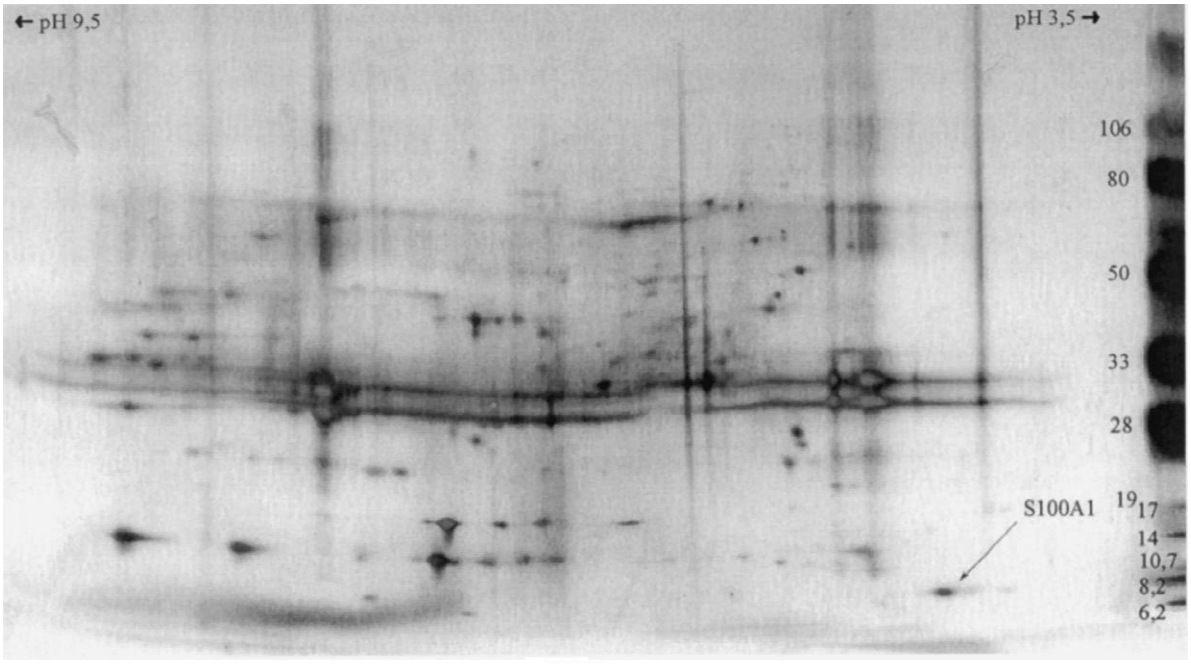
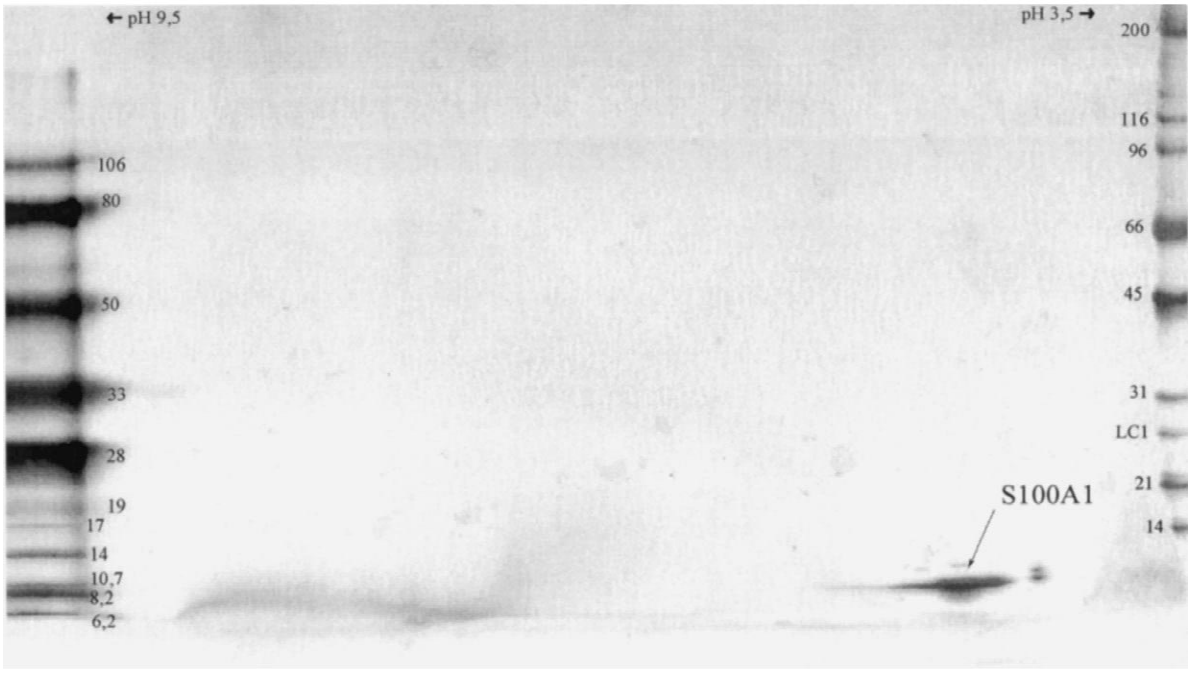


Fig. 5. (a) Silver stained SDS–PAGE of fractions from HIC and AIEX (Figs. 3 and 4): (1) fall through from Octyl-Sepharose, (2) Ca^{2+}-binding protein pool as eluting with EGTA from Octyl-Sepharose, (3–4) fall through from Q-Sepharose, (5) higher-molecular-mass proteins, (6) S100A1 monomer and homodimer, (7) calmodulin, (S) protein standards (left: Bio-Rad, Hercules, CA, USA; right: Merck, Darmstadt, Germany). (b) Western blot of fractions 1–7 from HIC and AIEX (Figs. 1 and 2): (1) no S100A1 immunoreactivity is detectable in the fallthrough from HIC, (2) (5) (6) S100A1 immunoreactivity. (S) biotinylated protein standards.



(a)



(b)

Fig. 6. (a) Silver stained two-dimensional PAGE analysis of porcine EDTA extract of myocardium. S100A1 is marked by an arrow. (b) Silver stained two-dimensional PAGE of purified porcine S100A1 after Q-Sepharose chromatography. First dimension immobiline IEF pH 3.5–9.5, second dimension SDS–PAGE 8–18% acrylamide. Mixture of protein standards from Bio-Rad (prestained low) and Merck (1700–17 000). S100A1 is marked by an arrow.

porcine myocardium, the purified native porcine S100A1 is shown as a single band in Fig. 6b that is located near to the calculated position for human S100A1with a M_r of 10 415 and an isoelectric point (p*I*) of 4.4. Other porcine S100 proteins with similar molecular masses but different p*I* however could not be detected [S100A4, (M_r 11 729; p*I* 5.9), S100A6 (M_r 10 180; p*I* 5.3) and S100C (M_r 11 180; p*I* 6.1)]. The yield was 1.4–2.0 mg purified protein per 100 g wet tissue.

Our purification strategy proved to be very effective in isolating human recombinant S100A1 from *E. coli*. Lanes 1–3 in Fig. 7 represent the ammonium sulfate supernatant of *E. coli* EDTA extract (1), the fall through of *E. coli* proteins after equilibrating the Octyl-Sepharose column with Ca^{2+} washing buffer (2) (elution profile not shown), and the EGTA elution peak (3) containing the human recombinant S100A1 with a purity of already more than 95% after this one-step HIC. Fig. 8 shows a characteristic elution profile of the following Q-Sepharose chromatography used for the final purification of S100A1. Lanes 4 and 5 (Fig. 7) are samples taken for analysis from the fall through peak (4–5) and lanes 6–8 (Fig. 7) taken from peaks 6–8 in Fig. 8 the latter

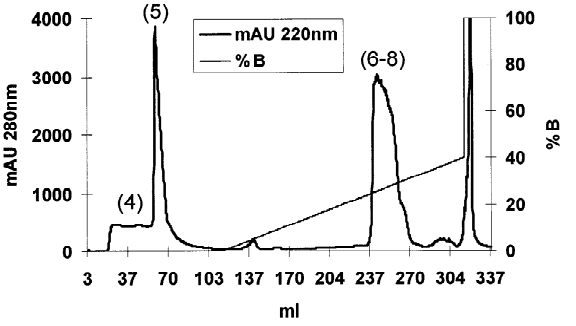


Fig. 8. Q-Sepharose anion-exchange chromatography of human recombinant S100A1 purified from recombinant *E. coli*: (4–5) fall through (6–8) recombinant S100A1.

representing the purified S100A1 fraction that again elutes with 300 m*M* NaCl. The yield of purified recombinant protein was 0.7–1.0 mg per 100 ml bacterial culture.

Both the native porcine as well as the human recombinant S100A1 protein fraction exerted a calcium dependent inhibitory effect on the phosphoglucomutase activity (Fig. 9) as it is described by Landar et al. [19]. At a given $CaCl_2$ concentration of 1 m*M* the enzyme activity was reduced in the presence of 1 m*M* S100A1 by 50% as compared to

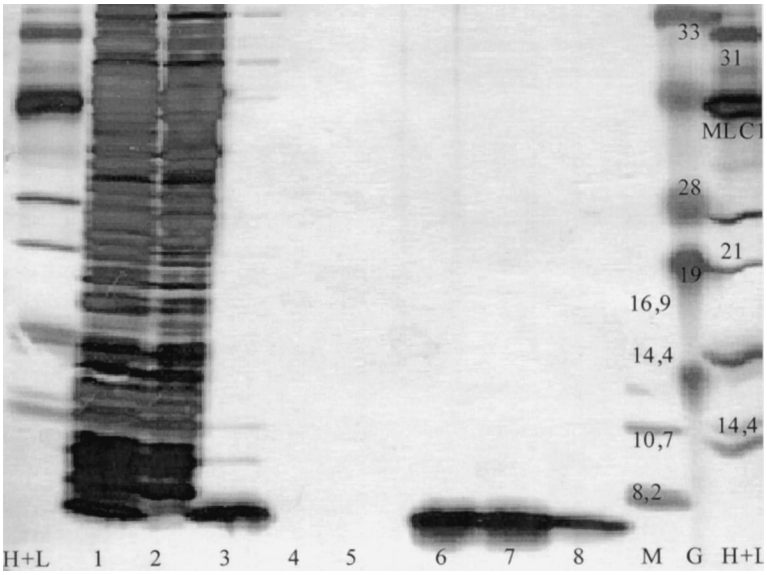


Fig. 7. SDS–PAGE of the purification steps for human recombinant S100A1: (1) EDTA extract from recombinant *E. coli*, (2) fall through from Octyl-Sepharose, (3) EGTA eluate from Octyl-Sepharose, (4) and (5) are samples from the fall through from Q-Sepharose (Fig. 8) after application of sample (3). (6) (7) and (8) represent consecutive samples from peak II (Fig. 8).

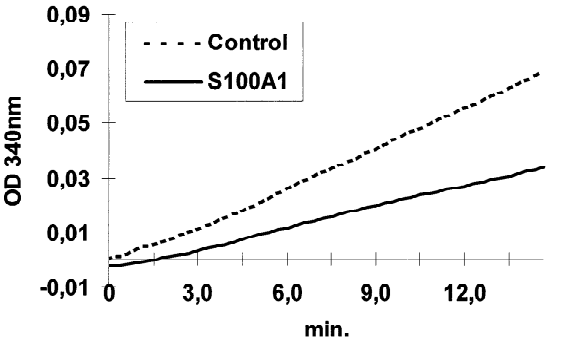


Fig. 9. Phosphoglucomutase assay according to Landar et al. [19]: phosphoglucomutase activity is inhibited by 50% in the presence of 1 m*M* $CaCl_2$ and 1 μ*M* human recombinant S100A1.

controls. Recently we reported about the modulation of the caffeine induced Ca^{2+} release by human recombinant S100A1 in saponine skinned skeletal muscle fibers of the rat leading to a significant increase of force transients [21]. Thus our protein purification procedure yields a highly purified S100A1 protein fraction that is biologically active both in biochemical as well as in physiological experiments.

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