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Heme ladder, a direct molecular weight marker for immunoblot analysis

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ABSTRACT

Detection methods for immunoblot analysis are often based on peroxidase conjugates. However, molecular weight markers directly detectable for general use in such systems are not available. Here, we describe the preparation of a direct molecular weight marker consisting of heme-tagged proteins, whose enzymatic activities make them detectable simultaneously with the antigen in peroxidase-based immunoblot systems. The peroxidase activity results from the covalent attachment of heme to selected engineered periplasmic proteins, catalyzed by the cytochrome *c* maturation system of *Escherichia coli*. The newly designed heme-tagged proteins were combined with a previously constructed heme-tagged maltose-binding protein and cytochrome *c*. The resulting heme ladder was shown to be suitable as a protein standard for direct molecular weight estimation in immunoblot analysis due to the peroxidase activity of its constituents. The heme ladder consists of proteins between 12 and 85 kDa and can be produced at low cost. The marker was stable when kept at 4, −20, and −80 °C for >6 months.

Protein molecular weight markers are widely used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[2] and immunoblot analysis. In the latter technique, proteins are often detected by enhanced chemiluminescence (ECL) of secondary antibodies that are conjugated to horseradish peroxidase (HRP) [1]. However, common protein standards are not detectable by ECL and therefore, no direct molecular weight estimation is possible. Nevertheless, there exist protein standards that can be detected on SDS-PAGE gels as well as after immunoblotting on nitrocellulose membranes, e.g., DualVue (Amersham Biosciences, Uppsala, Sweden), MagicMark (Invitrogen, Carlsbad, CA, USA), Pierce Chemiluminescent Blue Prestained Peroxidase-Labeled Protein Molecular Weight Marker (Pierce Biotechnology, Rockford, USA), and NexusWestern Precise Western Blot Marker (Bionexus, Oakland, CA, USA). However, these markers require special handling precautions and are expensive. Hence, there is a need for a better, simpler and cheaper protein ladder for direct molecular weight estimation in immunoblot analysis.

Heme is an iron-containing cofactor that is involved in a variety of cellular processes such as electron transport, enzymatic catalysis, or oxygen transport. In cytochromes, heme is bound covalently to the amino acid sequence Cys-Xaa-Yaa-Cys-His and acts as the redox-active center of the protein. Covalent heme attachment results in two thioether bonds, which are generated as an outcome of the reaction of thiol groups of the cysteine residues with the vinyl groups of heme. The histidine residue serves as an axial ligand to the heme iron [2]. Bacterial *c*-type cytochromes harbor an N-terminal signal sequence for export to the periplasm by the general protein type II secretion system (Sec) [3]. On the periplasmic side of the cytoplasmic membrane, heme is attached covalently to the apo-cytochrome *c* (non-heme-bound), resulting in holo-cytochrome *c* (heme-bound) [4]. Maturation of *c*-type cytochromes in *Escherichia coli* is a complex process requiring eight proteins CcmA-H encoded by the *ccmABCDEFGH* operon [5–7]. However, genomic expression of the *ccm* genes is only induced under anaerobic growth conditions [8] when *c*-type cytochromes are used for respiratory electron transport. By using a *ccmABCDEFGH* deletion mutant of *E. coli* (EC06) and a plasmid that expresses the *E. coli* cytochrome *c* maturation genes constitutively (pEC86), *c*-type cytochromes can be overexpressed independently of genomic regulation mechanisms [5,9]. Recently, the minimal sequence requirements for heme insertion into a cytochrome polypeptide were determined [10], thus allowing the development of a short heme tag CXXCHHHHHH for the covalent attachment of heme to the C-terminus of a protein lacking the ability to bind heme [11]. The resulting heme-tagged protein (heme-tagged maltose-binding protein, MBP*), referred to as artificial *c*-type cytochrome, was described to show intrinsic peroxidase activity [11].

Here, we describe a novel molecular weight marker that is detectable in all peroxidase-based immunoblot systems. Artificial *c*-type cytochromes of different molecular weights were produced. A mixture of these heme-tagged proteins resulted in a molecular weight marker with peroxidase activity that we have named heme

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[2] *Abbreviations used:* ECL, enhanced chemiluminescence; GlDH, glutamate dehydrogenase; HRP, horseradish peroxidise; MBP, maltose-binding protein; RBP, ribose-binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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ladder. This marker is visible on an SDS-polyacrylamide gel on common staining with Coomassie or similar methods. Additionally, it is detectable by a peroxidase activity staining either in a gel or on a nitrocellulose membrane and can thus be used for direct molecular weight estimation of a target protein by ECL detection.

Materials and methods

Strains, plasmids, and growth conditions

E. coli strain DH5α [12] was used for cloning. Heme-tagged proteins were expressed in *E. coli* EC06 (Δ*ccmA-H*) [5], a derivative of the K12-derivative MC1061 [13], harboring pEC86, a pACYC184 derivative that contains the *ccm* genes downstream of the *tet* promoter [9]. Plasmid pMP295 [11], a pMal-p derivative encoding the microperoxidase MP295, was used for expression of heme-tagged maltose-binding protein. Plasmid pMP379 was constructed from pMP251, a pISC2 [8] derivate where the *iciA* gene had been replaced by a sequence encoding a periplasmic heme peptide fusion, with 5′-G*CGGCCG*CGTGCCTGGCCTGCCACCATCACCATCACC AT-3′ encoding the short His_5-tagged heme peptide (QDAAACLACHH HHHH) fused to the *Bradyrhizobium japonicum* cytochrome c_{550} signal peptide [10] and comprising an *Eag*I restriction site (italics). The multiple cloning site of pBluescript II SK (+) was cloned subsequently into the *Eag*I restriction site of pMP251, leading to pMP379. The *Nde*I and *Xba*I cloning sites of this plasmid were used for the construction of heme-tagged proteins as depicted in Fig. 1.

Final antibiotic concentrations were 100 μg ml^{-1} for ampicillin and 10 μg ml^{-1} for chloramphenicol. For expression of proteins 200 ml of Luria-Bertani broth supplemented with trace metals (1 mM $CaCO_3$, 0.7 mM $FeCl_3$, 80 μM $MnCl_2$, 6 μM $CuSO_4$, 11 μM $CoCl_2$, 60 μM $ZnSO_4$, 50 μM H_3BO_3, 89 μM Na_2MoO_4, 2 mM EDTA in 5.5 mM HCl) was inoculated 1:100 with an overnight culture of the appropriate strain at 37 °C. Cultures were grown to an optical density of A_{600} = 0.8 and induced with 0.5% arabinose (heme-tagged OsmY and heme-tagged RBP) or 0.2 mM IPTG (heme-tagged MBP). At the time of induction the heme precursor δ-ALA (5-aminolevulinic acid hydrochloride) (Sigma, Buchs, Switzerland) was added at a concentration of 0.5 mM. Cells were harvested 3 h after induction by centrifugation at 6000*g*.

Plasmid construction

Plasmids encoding heme-tagged proteins are derived from pMP379. The *rbsB* gene was amplified with the primers rbSB_fw_1 (5′-GCACATATGAACATGAAAAAACTGGCTACC-3′) and rbSB_rv_1 (5′-GCATCTAGACTGCTTAACAACCAGTTTCAG-3′), and the *osmY* gene was amplified with the primers osmY_fw_1 (5′-GCACATAT GACTATGACAAGACTGAAG-3′) and osmY_rv_1 (5′-GCATCTAGACT TAGTTTTCAGATCATTTTTAACG-3′). The *Nde*I/*Xba*I DNA fragments encoding the RBP and the OsmY proteins were cloned into the corresponding sites of pMP379 resulting in the plasmids pSAH1 and pSAH2 encoding heme-tagged RBP (RBP*) and heme-tagged OsmY (OsmY*), respectively. The two constructs were confirmed by DNA sequencing with primers pJet 1.2 forward sequencing primer (5′-CGACTCACTATAGGGAGAGCGGC-3′) and pJet 1.2 reverse sequencing primer (5′-AAGAACATCGATTTTCCATGGCAG-3′) (Microsynth, Balgach, Switzerland).

Protein extraction and purification

Periplasmic proteins were obtained by cold osmotic shock treatment. The cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C, washed in cold 150 mM NaCl, and resuspended in cold 200 mM Tris-HCl, pH 8.5, 100 mM EDTA, 250 g L^{-1} sucrose, including protease inhibitors (Roche, Basel, Switzerland). The suspension was stirred for 20 min at 4 °C and centrifuged at 10,000*g* for 20 min. The pellet was resuspended in 10 mM Tris-HCl, pH 8.5, including protease inhibitors, stirred for 2 h at 4 °C, and centrifuged at 10,000*g* for 20 min. The supernatant contained the periplasmic proteins. His_6-tagged proteins were purified by Ni-NTA affinity chromatography (GE Healthcare, Uppsala, Sweden). Beads were washed with 10 bed volumes 50 mM Tris-HCl, pH 8, 300 mM NaCl, 20 mM imidazole and proteins were eluted with 8 bed volumes 50 mM Tris-HCl, pH 8, 300 mM NaCl, 300 mM imidazole. Maltose-binding proteins were purified by affinity chromatography on amylose resin (New England BioLabs, Frankfurt, Germany). Beads were washed with 5 bed volumes of 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA and proteins were eluted with 5 bed volumes of 20 mM Tris-HCl, pH 8, 200 mM NaCl, 1 mM EDTA, 10 mM maltose. The eluted proteins were stored in 10 mM Tris-HCl, pH 7.5.

Analytical methods

Native proteins with covalently bound heme were separated by SDS-PAGE (16%) [14] in the absence of a reducing agent (β-mercaptoethanol) and analyzed by the peroxidase activity stain (heme stain) as described previously [11]. Alternatively, proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes (Amersham Biosciences/GE Healthcare, Uppsala, Sweden) and then further detected directly or after immunoblot analysis by enhanced chemiluminescence (ECL). Immunoblot analysis of glutamate dehydrogenase from beef liver (GlDH) (Roche Diagnostics, Mannheim, Germany) with rabbit anti-glutamate dehydrogenase 1 (anti-GLUD1) (Acris Antibodies GmbH, Herford, Germany) at a dilution 1:3000 was carried out by standard methods [15]. Peroxidase-labeled conjugate goat anti-rabbit immunoglobulin G (heavy plus light chains) (Calbiochem, Merck KgaA, Darmstadt, Germany) at a dilution of 1:40,000 was used for the visualization of glutamate dehydrogenase. For ECL, blotted proteins were washed twice with PBS for 10 min. The membrane was covered with SuperSignal West Dura (Thermo Scientific, Waltham, MA, USA) and incubated for 5

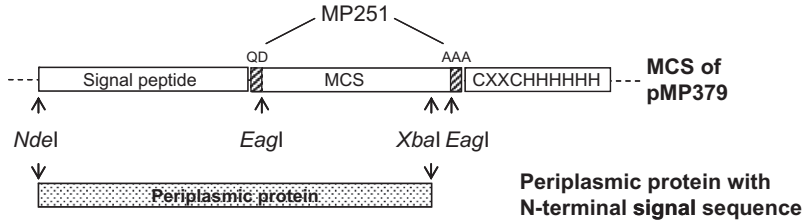

Fig.1. Cloning strategy for heme-tagged proteins. Plasmid pMP379 harboring a gene encoding an N-terminal signal peptide followed by a multiple cloning site (MCS) and a short His-tagged heme peptide. The sequence encoding the microperoxidase MP251 [10] was disrupted by inserting the multiple cloning site of pBluescript II SK (+) into an *Eag*I site. The *Nde*I/*Xba*I cloning site was used for the construction of heme-tagged proteins. The backbone of the pISC-2 vector [8] is indicated as a dashed line. Restriction sites for *Nde*I, *Eag*I, and *Xba*I are indicated by vertical arrows.