Biochimica et Biophysica Acta 1830 (2013) 2946–2953

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Stability and iron oxidation properties of a novel homopolymeric plant ferritin from adzuki bean seeds: A comparative analysis with recombinant soybean seed H-1 chain ferritin

Meiliang Li, Shaojun Yun, Xiuli Yang, Guanghua Zhao *

CAU & ACC Joint-Laboratory of Space Food, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing Key Laboratory of Functional Food from Plant Resources, Beijing 100083, China

ARTICLE INFO

Article history:
Received 29 September 2012
Received in revised form 3 January 2013
Accepted 4 January 2013
Available online 11 January 2013

Keywords:
Plant ferritin
Extension peptide (EP)
Adzuki bean seed
Homopolymer
Iron oxidation

ABSTRACT

Background: All reported plant ferritins are heteropolymers comprising two different H-type subunits. Whether or not homopolymeric plant ferritin occurs in nature is an open question.
Methods: A homopolymeric phytoferritin from adzuki bean seeds (ASF) was obtained by various protein purification techniques for the first time, which shares the highest identity (89.6%) with soybean seed H-1 ferritin (rH-1). Therefore, we compared iron oxidation activity and protein stability of ASF with those of rH-1 by stopped-flow combined with light scattering or UV/Vis spectrophotography, SDS- and native- PAGE analyses. Additionally, a new rH-1 variant (S68E) was prepared by site-directed mutagenesis approach to elucidate their difference in protein stability.
Results: At high iron loading of protein, the extension peptide (EP) of plant ferritin was involved in iron oxidation, and the EP of ASF exhibited a much stronger iron oxidative activity than that of rH-1. Besides, ASF is more stable than rH-1 during storage, which is ascribed to one amino acid residue, Ser68.
Conclusions: ASF exhibits a different mechanism in iron oxidation from rH-1 at high iron loading of protein, and a higher stability than rH-1. These differences are mainly stemmed from their different EP sequences.
General significance: This work demonstrates that plant cells have evolved the EP of phytoferritin to control iron chemistry and protein stability by exerting a fine tuning of its amino acid sequence.

1. Introduction

Ferritins are a special class of diiron proteins that play a role in both iron housekeeping and iron detoxification. The importance of these proteins is reflected by their wide distribution throughout the animal, plant, and microbial kingdoms. Animal ferritins are generally heteropolymers composed of 24 subunits of two types, namely H and L subunit, which assemble into a nearly spherical shell characterized by a 432-point symmetry. The two types have about 55% identity in amino acid sequence. The ratio of the two kinds of subunits in the native protein varies according to the nature and the function of the tissues [1,2]. The H-type subunit of vertebrate ferritins contains a dinuclear iron "ferroxidase site" at which the rapid oxidation of Fe^{2+} to Fe^{3+} by dioxygen or hydrogen peroxide occurs; subsequent hydrolysis of the Fe^{3+} and its migration away from the ferroxidase site leads to formation of the initial Fe^{3+} mineral core within the inner cavity. Unlike the H-chain, the acidic L-subunit lacks the ferroxidase center. Ferritins from lower vertebrates, such as bullfrogs and fish, contain a third subunit type, named the M (H′) chain, which harbors the residues forming both the ferroxidase center and the nucleation site [3]. In contrast, two different types of ferritins are found in bacteria, often within the same cell. Although the two types of ferritins from bacterial are chemically, structurally, and functionally different, they are homopolymers consisting of one H-type subunit [1,4].

Different from animal and bacterial ferritins, plant ferritin exhibits two distinctive structural features. Plant ferritin is only composed of H-type subunits, but each H-type subunit in mature plant ferritin contains a specific extension peptide (EP) at its N-terminal sequence. This represents the first difference in structure. In the case of soybean seed ferritin (SSF), each EP domain is composed of about 30 amino acid residues [5–7]. The crystal structure of recombinant soybean seed H-4 ferritin (rH-4) shows that the EP is located on the exterior surface of protein and stabilizes the entire oligomeric conformation of plant ferritin by its interaction with a neighboring subunit on the shell surface [8]. Recent studies of our group reveal the role of the EP during iron oxidative deposition in plant ferritin as the second binding and ferroxidase

Abbreviations: ASF, plant ferritin from adzuki bean seed; SSF, soybean seed ferritin; nSSF, naturally occurring soybean seed ferritin; apoSSF, apo-soybean seed ferritin; PSF, pea seed ferritin; EP, extension peptidel; EP-a, extension peptide of ASF; EP-1, extension peptide of H-1 subunit of soybean seed ferritin; EP-2, extension peptide of H-2 subunit of soybean seed ferritin; EP-3, extension peptide of H-3 subunit of soybean seed ferritin; EP-4, extension peptide of H-4 subunit of soybean seed ferritin; EP-5, extension peptide of H-5 subunit of soybean seed ferritin; rH-2, recombinant soybean seed H-2 chain ferritin; rH-1, recombinant soybean seed H-1 chain ferritin; DLS, dynamic light scattering

* Corresponding author. Tel./fax: +86 10 62738737.
E-mail address: gzhao1000@163.com (G. Zhao).

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.01.004

center that plays an important role in iron oxidation at high iron loading of ferritin (>48 irons/shell) [9]. On the other hand, the EP exhibits a significant serine protease-like activity, which is responsible for protein auto-degradation during seed germination. Associated with the degradation is faster iron release from ferritin to meet the requirements of seedling growth [10]. Another major difference between plant ferritin and other ferritins in structure is that, so far, all known plant ferritin has been found to be a heteropolymer usually composed of two different H-type subunits [11]. Whether or not homopolymeric plant ferritin occurs in nature is an open question.

In this study, a novel homopolymeric plant ferritin — adzuki bean seeds ferritin (ASF) was for the first time isolated from adzuki bean seeds and purified to homogeneity, which only consists of one kind of H-type subunit. This subunit shows the most similarity (~90%) to the H-1 of SSF. However, this new protein exhibits a significantly different mechanism in iron oxidation from its analog, rH-1. For example, at moderate iron loading ($48<Fe^{2+}$/protein shell <200), iron oxidation was dominated by the diiron ferroxidase center of rH-1, while this situation is greatly changed in ASF; instead, the EP, a specific domain located on the exterior surface of plant ferritin rather than its animal counterpart, plays an important role in iron oxidation. At high iron loading (>200 Fe^{2+}/protein shell), the EP is involved in iron oxidation in these two ferritins. Additionally, the EPs have different effects on their stability. These new findings emphasized the importance of the EP in the iron oxidation and protein stability.

2. Materials and methods

2.1. ASF isolation and purification

Approximately 1 kg of adzuki bean seeds was soaked in distilled water overnight, decorticated and blended in two volumes of extraction buffer (50 mM KH_2PO_4–Na_2HPO_4, pH 7.0, 1% PVP), filtered through cheesecloth and the residue was removed. The filtrate was centrifuged at 10,000 ×*g* for 10 min to separate the insoluble material. The supernatant was adjusted to 300 mM $MgCl_2$, stored for 60 min at 4 °C followed by addition of sodium citrate to final concentration of 450 mM to complex the magnesium. After 8 h, this supernatant was centrifuged at 12,000 ×*g* for 35 min at 4 °C. The brown pellet thus obtained was dissolved in 50 mM Tris–HCl buffer (pH 9.0), and was dialyzed against the same buffer three times. The protein was further purified by ion exchange chromatography (IEC) in a DEAE Sepharose Fast Flow column previously equilibrated in 50 mM Tris–HCl buffer (pH 9.0). Fractions containing ASF were pooled and concentrated, and finally loaded to Sephacryl S-300 gel filtration chromatography (GFC) equilibrated before use in 50 mM Tris–HCl buffer (pH 9.0, 0.15 M NaCl).

2.2. Polyacrylamide gel electrophoresis

The molecular weight of the native ASF was estimated by PAGE using a 4–20% polyacrylamide gradient gel run at 25 V for 14 h at 4 °C employing Tris–HCl (25 mM, pH 8.3) as running buffer. Gels were stained with Coomassie brilliant blue R250. Gel electrophoresis under denaturing conditions was carried out with 15% polyacrylamide-SDS gel as reported by Laemmli [12]. Protein samples (~20 μg) were suspended in 50 μL of water. To the solution were added 100 μL of sample buffer containing 25% glycerol, 12.5% 0.5 M Tris–HCl, pH 6.8, 2% SDS, 1% bromophenol blue and 5% β-mercaptoethanol. All quoted concentrations were final concentrations. After the solution was boiled for 10 min, the supernatant was isolated by centrifugation at 10,000 *g* for 10 min.

2.3. NH_2-terminal amino sequence analysis

The amino acid sequence of the N-terminus was determined on a protein sequencer (Applied Biosystems Procise-PROCISE 491) using automated Edman degradation. After SDS-PAGE, the protein was transferred to a polyvinylidene difluoride membrane (Millipore) and stained with Coomassie Brilliant Blue R250. The subunit was eluted from the membrane and the sequence of the 15 amino acids at the NH_2-terminus was determined.

2.4. In-gel trypsin digestion and liquid chromatography–tandem mass spectroscopy (LC–MS/MS)

Purified protein was excised from a 15% SDS-polyacrylamide gel and dissolved in 100 μL destaining solution of 50 mM NH_4HCO_3 in 50% acetonitrile. The sample was incubated until the blue color of the gel was completely removed. Then, the gel was dried for 20 min and 10 μL of trypsin solution (0.01 mg/mL in 25 mM NH_4HCO_3, pH 8.0) was added. After incubation at 37 °C overnight, the peptides were recovered by incubating with 50 μL of extraction solution (50% acetonitrile/0.1% trifluoroacetic acid) for 30 min. The extracted peptide solution was dried and dissolved in 0.1% formic acid and analyzed by LC–MS/MS (Agilent, USA). Peptides were switched onto a Symmetry C18 column using a mobile phase containing 0.1% formic acid and 0%–40% acetonitrile gradient over 75 min at a 0.2 mL/min flow rate. MS/MS spectral data were collected for the m/z range 50–2600. A protein database search was performed against the SWISS-PROT and NCBI-nr database using the MASCOT search engine (http://www.matrixscience.com) with one missed cleavage site.

2.5. cDNA cloning of ASF

Total RNA was prepared from tender leaves of adzuki bean using Sepasol RNA I (Nacalai Tesque, http://www.nacalai.com/), according to the manufacturer's instructions, and then stored at −80 °C for further use. First-strand cDNA was synthesized using AMV reverse transcriptase (Takara, http://www.takara-bio.com/) with 1.5 μg total RNA and oligo (dT) adaptor primer containing the sequence of the M13M4 universal primer. The resulting first-strand cDNA was used as a template for PCR amplification of a partial sequence of ASF and for 3′-RACE PCR using the M13M4 primer and degenerate primer 1 (TACTTTGACAGGGAC AACG). Nested PCR was performed using the M13M4 primer and degenerate primer 2 (GGATCCGGTACCTCTAGATCAG). PCR was performed using Blend Taq (Toyobo, http://www.toyobo-global.com/). A 636 bp fragment of the nested PCR product was excised from the gel and cloned into the pCR2.1 vector of the TOPOTA cloning kit (Invitrogen, http://www.invitrogen.com/). The DNA sequence of the cloned DNA fragment was verified by automated DNA sequencing (ABI PRISM 3100, Applied Biosystems, http://www.appliedbiosystems.com/).

5′-RACE was performed using a 5′-full RACE core set (Takara) according to the manufacturer's protocol. Reverse transcription was performed using the 5′-phosphorylated RT primer (CTGCCACACTGT), Prime script RTase (Takara) and total RNA of adzuki bean. The resulting first-strand cDNA was self-ligated using RNA ligase to make circular DNA, and was used as a template for the PCR reaction. PCR and nested PCR amplification were performed using primers Sense 1 (5′-TCTTCCA CCACGAGTGTTCTG-3′) and Antisense 1 (5′-TGTGGAAAAGGGGGATGC-3′), and primers Sense 2 (5′-TTGGAAAGCTACGTTGTC-3′) and Antisense 2 (5′-AGAAGTTGGTGAATGAG-3′), respectively. The nested PCR product was excised from the gel and cloned into the T-vector (Takara, http://www.takara-bio.com/).

2.6. Stopped-flow light scattering experiments

Stopped-flow light scattering measurement experiments were performed with a Hi-Tech SFA-20 M apparatus in conjunction with a Cary Eclipse spectrofluorimeter (Varian) as recently described [10]. Both excitation and emission wavelengths were set to 450 nm, and the time-dependent change in scattering light was set to a 90° angle, perpendicular to the beam, and recorded as previously reported. All