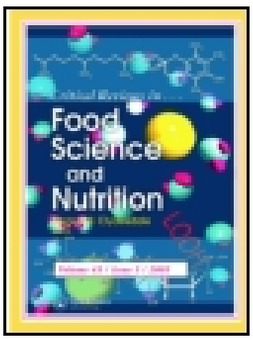

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REVIEW

Advancements of nature nanocage protein: preparation, identification and multiple applications of ferritins

Han Li[a], Xiaoyu Xia[a], Xiaoyi Tan[b], Jiachen Zang[b], Zhenyu Wang[a], Hesham R. El-Seedi[c], and Ming Du[a]

[a]School of Food Science and Technology, National Engineering Research Center of Seafood, Collaborative Innovation Center of Seafood Deep Processing, Dalian Polytechnic University, Dalian, China; [b]College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China; [c]Department of Medicinal Chemistry, Biomedical Centre, Uppsala University, Uppsala, Sweden

ABSTRACT

Ferritin is an important iron storage protein, which is widely existed in all forms of life. Ferritin can regulate iron homeostasis when iron ions are lacking or enriched in the body, so as to avoid iron deficiency diseases and iron poisoning. Ferritin presents a hollow nanocage, which can store ions or other small molecular substances in the cavity. Therefore, ferritin shows its potential as a functional nanomaterial that can deliver nutrients or drugs in a targeted manner to improve bioavailability. Due to the special structure, the research on ferritin has attracted more and more attention in recent years. In this paper, the structural characteristics of ferritin were introduced, and the natural purification and prokaryotic expression methods of ferritin from different sources were described. At the same time, ferritin can bind to small molecules, so that it has the activity of small molecules, to construct a new type of ferritin. As a result, ferritin plays an important role as a nutrient substance, in targeted transport, and disease monitoring, etc. In conclusion, the yield of ferritin can be improved by means of molecular biology. Meanwhile, molecular modification can be used to make ferritin have unique activity and function, which lays a foundation for subsequent research.

HIGHLIGHTS

1. The molecular and structural properties of ferritins were clearly described.
2. Isolation and purification technologies of ferritin were compared.
3. Characterization, functions and molecular modifications mechanism of ferritin were reviewed.
4. The applications of ferritin in pharmaceutical and food industry were prospected.

KEYWORDS

Ferritin; structure characterization; prokaryotic expression; bioactivity; applications

Introduction

Iron is one of the most important basic elements in organisms. It can directly affect the tricarboxylic acid cycle, electron transport, nitrogen fixation, DNA synthesis and detoxification reaction, thus affecting the vital cascades of organism̀s metabolism (Andrews 1998). Fe^{2+} can be oxidized to an insoluble form of Fe^{3+} under the oxygen-rich conditions, it may be one of the reasons that cause the amount of free and available iron ions decreases, so it has the disadvantages of low bioavailability. The total content of iron in human body is about 3–5 g, except for hemoglobin, the rest of the iron is generally present in the form of ferritin (Hentze, Muckenthaler, and Andrews 2004; Crichton and Charloteaux-Wauters 1987; Theil 2004). In 1884, Schmiedeber was the first to find a water-soluble iron storage protein (Theil and Goss 2009).

In recent years, the isolation, purification and application of ferritin have become a hot topic. At present, there are many researches on ferritin from plants, especially ferritin from seeds. But the fact that the ferritin of animal sources is low and mainly exists in the metabolically active tissues such as spleen and liver, makes the rapid and efficient isolation and purification of ferritin particularly important and crucially needed (Song et al. 2017). Therefore, the article gives an introduction to the structural characteristics, isolation, purification and applications of ferritin, providing a theoretical basis for the industrial development and health values of ferritin (Harrison and Arosio 1996).

Distribution of ferritin

Ferritin is a highly conserved protein, where the amounts, proportions and compositions of its subunits vary significantly in different organisms as illustrated in Table 1. As shown in Table 1, animal ferritin, plant ferritin and some microbial ferritin share relatively a similar conservative structure, but the similarity of amino acid sequences is low except for the amino acid at the oxidation center (Andrews 2010). Proteins that regulate different DNA functions and metabolism and are fixed to an action site by recognizing

CONTACT Ming Du duming@dlpu.edu.cn School of Food Science and Technology, National Engineering Research Center of Seafood, Dalian Polytechnic University, Dalian 116034, China

Table 1. Distribution of ferritin from different source.

No.	Ferritin/Subunit type	Source	Sample	PDB ID	Sequence length	Reference
1	Animal ferritin	Animal	Chaetopterus variopedatus	5WPN	171	(De Meulenaere et al., 2017)
2	Plant ferritin	Plant	Glycine max	3A9Q	212	(Masuda et al., 2010)
3	Bacterial ferritin	Bacteria, archaea, viruses, fungi	Archaeoglobus fulgidus	3KX9	173	(Sana et al., 2013)
4	Dps	Bacteria, archaea, viruses, plants	Thermosynechococcus elongatus BP-1	2C41	158	(Franceschini et al., 2006)
5	Rubrerythrin	Bacteria, archaea, animals, fungi	Desulfovibrio vulgaris str. Hildenborough	1RYT	190	(Demaré et al., 1996)
6	Encapsulin	Bacteria, archaea	Quasibacillus thermotolerans	6N63	198	(Giessen et al., 2019)
7	L-chain	Mammals (humans, pigs, horses, etc)	Equus caballus	1AEW	174	(Hempstead et al., 1997)
8	H-chain	Mammals (humans, pigs, horses, etc.), plants (soybeans, chickpeas, black beans, etc.), shellfish (oysters, clams, mussels, etc.), fish (grass carp, fragrant fish, salmon, etc.)	Homo sapiens	6J4A	183	(Zhang et al., 2019)
9	M-chain	Fish (Trematomus newnesi, goby burgess, etc.)	Lithobates catesbeianus	4DAS	176	(Bertini et al., 2012)

certain DNA sequences, the proteins are called DNA-binding proteins (Dps), which exists in bacteria and other organisms storing iron ions, can also bind to DNA in extreme environments, protecting DNA from damage (Chiancone and Ceci 2010; Zeth 2012). It is composed of only 12 subunits to form a 9 nm protein structure with a maximum iron ion capacity of 500 in each Dps. Rubrerythrin (Rr) is a non-heme ferritin isolated from anaerobic sulfuric acid reducing bacteria. It presents in the bacteria as homodimer with two different metal centers, with a molecular weight of about 44,000 Da (Bonomi, Kurtz, and Cui 1996). Encapsulin is one of the largest ferritin exists in bacteria without ferrous oxidation center, which can store about 30000 iron ions, and the oxidation ability of Encapsulin was determined by some enzymes of internal cavity. Bacteria depend on a well-regulated iron homeostasis to survive in an adverse environment. A key component of the iron homeostasis mechanism is the localization of Fe^{3+} in bacterial ferritin, and subsequent mobilization in the form of Fe^{2+} to meet metabolic requirements (Rahmanpour and Bugg 2015).

Molecular properties of ferritin

Structure of ferritin

Ferritin is a kind of macro-molecular protein, which was shown symmetrization structure as a whole and present spherical structure. Ferritin consists of a hollow protein shell with an iron core in the center. The iron core is about 8 nm in diameter, and the outer diameter of the ferritin is about 12 nm. The iron core consists of iron hydroxide and phosphate, and the different species may vary in the size and the composition of the iron core (Harrison and Arosio 1996; Lawson et al. 1991). The ferritin cavity can store up to 4500 Fe^{3+}. Research has shown that, ferritin is made up of 24 approximately cylindrical subunits with same structure (except for bacteria, *Listeria innocua*) (Ford et al. 1984). The subunits are all composed of four α-helix bundles (A, B, C, D) and a short helix (E). There is a BC-loop connecting the two amino groups in the BC helix, and the E helix is located at the end of the four α-helix bundles at the angle of 60° (Chasteen and Harrison 1999; Harrison et al. 1996). The subunit is shown in Figure 1a (Pozzi et al. 2015). The 24 subunits of ferritin are assembled into a dodecahedral protein shell in a 432 symmetrical pattern by four-, three-, and two-fold symmetry, these four-, three-, and two-fold symmetry are acting as a natural protein channels that pump Fe^{2+}, oxygen or other molecules in and out of ferritin which acts as a link between the internal cavity of ferritin and the external environment. The crystal structure of ferritin is shown in Figure 1b,c (Pozzi et al. 2015). When the content of Fe^{2+} in cells increases, free radical can induce peroxidation and then lead to cell damage. Ferritin can catalyze the reaction between Fe^{2+} and Fe^{3+} and store Fe^{3+} in the cavity. When the Fe^{2+} in the cells was detected to be low, Fe^{3+} could be reduced to Fe^{2+} with the help of reductants, Fe^{2+} could be released from the intracellular cavity for cell metabolism (Theil and Goss 2009). Because of its unique properties mentioned above, ferritin can be used as an effective iron supplement, as well as to remove the toxicity of iron ions and regulate the balance of iron metabolism in vivo (Laulhere, Laboure, and Briat 1989). Ferritin has remarkable stability, which can resist to high temperature (75 °C) and relatively extreme acidic and alkaline environment (pH $<$ 2.8 or pH $>$ 10.6). At the same time, the extreme pH environment will induce the spontaneous depolymerization of ferritin into single subunits, and the unique protein structure will disappear. However, when pH is adjusted to neutral, ferritin can be reassembled without structural change (Stefanini et al. 1996). Therefore, ferritin has good application value and development prospects to be used as a nanoscale material (Zhang et al. 2014).

Characterization of plant-derived ferritin

Plant-derived ferritin generally exists in plastids, such as amyloplast and chlorophyllinite. Plant-derived ferritin has only H subunit, and each H subunit has a ferroxidase site (Zhao 2010). Recent studies have shown that legume ferritin has two H subunits, namely H-1 subunit (26.5 kDa) and H-2 subunit (28.0 kDa), and the two H subunits have about 80% sequence similarity (Liao, Yun, and Zhao 2014). However, the proportions of the two H subunits are different in

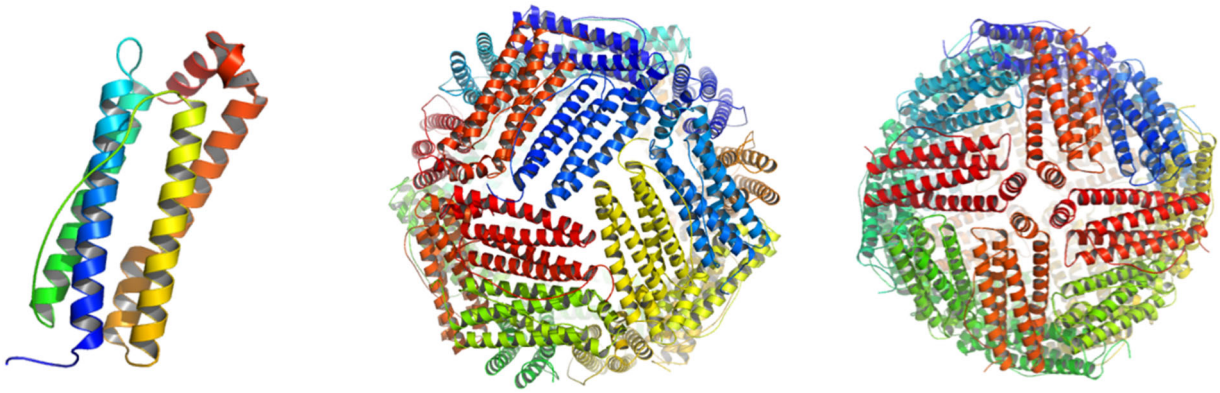

Figure 1. Structure of human ferritin subunit (a). The subunits are all composed of four α-helix bundles (A, B, C, D) and a short helix (E). There is a BC-loop connecting the two amino groups in the BC helix, and the E helix is located at the end of the four α-helix bundles at the angle of 60°. Crystal structure of ferritin at the three-fold channels (b) and four-fold channels (c). The 24 subunits of ferritin are assembled into a dodecahedral protein shell in a 432 symmetrical pattern by four-, three-, and two-fold symmetry, these four-, three-, and two-fold symmetry are acting as a natural protein channels that pump Fe^{2+}, oxygen or other in and out of ferritin which acts as a link between the internal cavity of ferritin and the external environment.

different sources of legumes, with soy ferritin 1:1, pea ferritin 1:2, black bean ferritin 2:1, and red bean and chickpea ferritin containing only H-1 subunit. The ferroxidase site of H-1 subunit is composed of Glul01, Hisl39, Glul34, Glul35, Tyrl07, Glul84, and Gln21, and the ferroxidase site of H-2 subunit is composed of Glul01, Hisl39, Glul34, Glul35, Tyrl08, Glul85, and Gln21 (Li, Hu, and Zhao 2009; Masuda et al. 2007). The function of H-1 subunit is to catalyze the oxidation of Fe^{2+}, and the H-2 subunit and EP domain work together to catalyze Fe^{2+}. The synergistic effect of the two subunits can accelerate the absorption of iron ion in plant cells (Li et al. 2013). Compared with animal-derived ferritin, plant-derived ferritin has two unique structures: transit peptide (TP) and extension peptide (EP). EP consists of about 30 amino acids, located at the N-terminal of the plant-derived ferritin subunit, extending to the outside of the ferritin structure, and can still form a 432 symmetrical pattern between the subunits. EP in legumes can not only interact with H-2 subunit, but also participate in the process of ferrous oxidation and precipitation. At the same time, due to the serine-like activity of the EP, it is possible to induce the occurrence of self-degradation during processing and storage (Yang et al. 2010). Moreover, for the channel structure, both the 3-fold channel and the 4-fold channel of plant-derived ferritin are hydrophilic channels (Lobreaux et al. 1992).

Characterization of animal-derived ferritin

Animal ferritin is mainly found in cytoplasm and usually consists of 24 heavy subunits (H subunit, 21,000 Da) and light subunit (L subunit, 19,500 Da), which have different functions and together participate in oxidizing sedimentation. There was a 55% similarity between the H and L subunit of the same species, while the amino acid sequence homology of the H subunit among different species was 88–99%, and that of the L subunit was 78–92% (Zhao, Arosio, and Chasteen 2006). Conserved amino acids (Glu27, Glu61, Glu62, His65, Glu107, Tyr134, Glnl41) are present in the H subunit, and the above amino acids act synergistically,

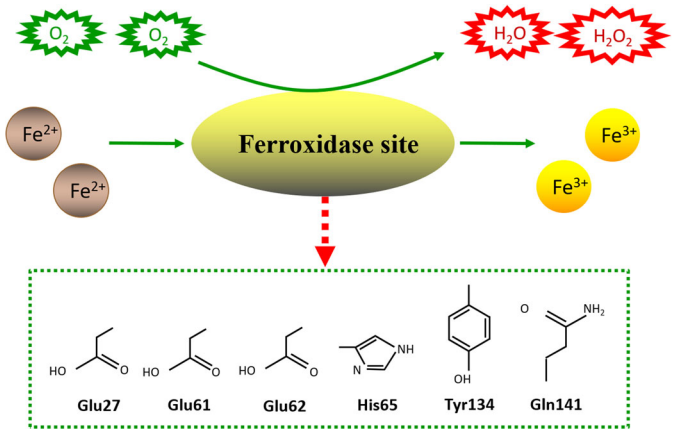

Figure 2. The catalytic mechanism of the ferroxidase center of ferritin. Ferroxidase site can catalyze the rapid oxidation reaction of Fe^{2+} to Fe^{3+} in the presence of oxygen through the combined action of 7 amino acids.

namely at the ferroxidase site, to catalyze the rapid oxidation of Fe^{2+}. Recent studies have shown that each ferrous oxidation center can catalyze two Fe^{2+}. However, H subunit can promote the slow oxidation of Fe^{2+} and form iron core of the nucleation site (Bou-Abdallah et al. 2008; Crichton et al. 1996; Toussaint et al. 2007). The catalytic process is shown in Figure 2. In different tissues or organs, the proportion of H and L subunit is different to meet different needs. H subunit usually exists in metabolic tissues such as brain and heart, while L subunit is found in the liver and spleen (Harrison and Arosio 1996; Orino et al. 2004). Recent studies have shown that hydrophilic amino acids, such as glutamate and glutamine, accumulate in the 3-fold channel of the ferritin, to transport Fe^{2+} into the ferroxidase site. Since there are many hydrophobic amino acids in the 4-fold channel, such as leucine, the hydrophobic molecules enter the ferroxidase site through diffusion, such as oxygen, etc.

In addition to the H and L subunit, a unique subunit has been found in lower vertebrates and named M subunit according to its electrophoretic mobility (Andrews et al. 1992; Dickey et al. 1987). M subunit also has nucleation site and has the ability of forming cavity. In bullfrogs, the H and M subunits are present in the metabolically active tissues,

while the H and L subunits are present in the liver (Carrondo 2003). The liver and spleen ferritin of *Trematomus newnesi* and *Trematomus bernacchii* was mainly composed of M/H subunit heteropolymers, which enabled the oxidation and mineralization of ferritin at low temperatures (Giorgi et al. 2008). Thus, it has been shown that H/L ferritin heteropolymers are required in mammals to efficiently oxidize and absorb iron ion, whereas in lower vertebrates the functions of oxidation and absorption of iron can be performed by M subunit homopolymers.

Preparation of ferritin

Purification of plant-derived ferritin

At present, the study of plant ferritin is done only in legumes, mainly derived from seeds of pea, soybean, chickpea, black bean, corn and alfalfa, which is composed of two subunits of 26.5 KDa and 28.0 KDa in different proportions. In 1962, Hyde et al. first observed ferritin in peas by electron microscopy, and then successfully purified ferritin from peas by soaking, homogenizing, filtering, co-precipitation, and chromatography (Hyde et al. 1963). The successful preparation of plant ferritin provides some reference for the subsequent purification of other plant ferritin. In 1972, Laulhere et al. improved the methods of isolation and purification of plant ferritin, the starch is first removed by centrifugation, the precipitate was obtained by high-speed centrifugation, and the crude ferritin was obtained after salting-out and purified by anion exchange column. The above methods successfully prepared large amounts of pure pea and soybean ferritin (Laulhere, Lescure, and Briat 1988). Further studies by Laulhere et al. found that ferritin could be obtained by $MgCl_2$ specifically, thus reducing plenty of centrifugal steps, and demonstrated that gel filtration column chromatography could replace anion exchange column chromatography for purification (Laulhere and Briat 1993). In 1995, Barcelo et al. purified ferritin from clover and proved its molecular weight to be about 560 KDa, confirming an iron core of 4 nm diameter and iron ion content of about 1300. Experimental results showed the ratio of H-1 subunit to H-2 subunit of soybean ferritin was 1:1, that of pea ferritin was 1:2, black bean ferritin was 2:1, adzuki bean and chickpea ferritin had only H-1 subunit (Li et al. 2013; Deng et al. 2010a). Previous studies suggested that H-1 subunit is formed by H-2 subunit through hydroxyl radical degradation (Li, Hu, and Zhao 2009). Recently, the peptide mass fingerprinting (PMF) of soybean, pea and chickpea ferritin subunits was compared, and it was found that the PMF of H-1 and H-2 were significantly different (Deng et al. 2010b; Lv, Liu, and Zhao 2014), and the coding genes of soybean ferritin H-1 and H-2 subunits were cloned as SFERH-1 and SFERH-2, respectively, proved that plant ferritin is encoded by multigene (Masuda, Goto, and Yoshihara 2001; Masuda et al. 2007).

Purification of animal-derived ferritin

In 1937, Laufberger first isolated and purified ferritin from vertebrate horses, which was proved to be a nontoxic protein that can store large amounts of iron ions, and subsequently purified and identified ferritin from vertebrate and invertebrate animals (Harrison and Arosio 1996). Worwood et al. used two methods to isolate and purify serum ferritin and proved that the effect of extraction by immunosorbent and ion exchange chromatography were significantly better than that by conventional heating and ion exchange chromatography (Worwood et al. 1976). Pagé et al. purified normal human liver ferritin using ultrafiltration, Sephacryl S-300 gel filtration and DEAE-Affi Gel Blue. The method utilized the thermal stability of ferritin and its large molecular weight, and each step of the purification process is highly repeatable (Pagé, Lagueux, and Gauthier 1980). Santos et al. isolated the ferritin of chicken spleen by salting out and Sephadex G-200, the purified ferritin was identified as a single band by electrophoresis (Santos Benito and Martin Mateo 1983). Cham et al. isolated and purified the ferritin of from human liver by a simple, rapid method of methanol and heat treatment, and the results of immunoelectrophoresis and protein content proved that the method did not affect the antigenicity of ferritin (Cham et al. 1985). Passaniti et al. purified chicken liver ferritin by gel filtration chromatography and immunoaffinity chromatography, respectively. The results showed that the ferritin obtained by immunoaffinity chromatography was basically free of impurities, and the purification method was faster and simpler (Passaniti and Roth 1989). Huang et al. purified stingray liver ferritin and the microstructure were characterized using transmission electron microscopy (TEM), and the homology of H subunits and L subunits was analyzed by peptide mass fingerprinting (Huang et al. 2009).

Heterogeneous expression of ferritin

Ferritin mainly exists in animal organs, the raw materials are expensive and the content is low, so the steps of obtaining natural ferritin are complicated, which was a great waste to material resources and manpower. Due to the low content and difficulty in obtaining, and with the rapid development of genetic engineering and molecular biology in recent years, the construction of recombinant expression vector and its efficient expression has gradually become one of the conventional extraction methods. Studies have shown that the ferritin subunits of many different species have been successfully cloned and expressed, such as mice, horses, humans and other mammals, higher plants such as peas and broad beans, and bacteria such as nitrogen-fixing bacteria.

The fusion ferritin (the combination of heavy and light chain ferritin) was expressed in recombinant *E.coli* and purified through two-step sonication and gel filtration chromatography by Yun et al (2012). The molecular weight of a single band was verified by electrophoresis to be 38 kDa. The method does not require heat treatment and salting out, and can be used for purification of ferritin in large quantities. The expression vector containing the ferritin gene from *Fenneropenaeus chinensis* and *Litopenaeus vanname* was successfully cloned by Zhang et al. and Zhou et al (Zhang et al. 2006). It was proved that the ferritin of shrimp was

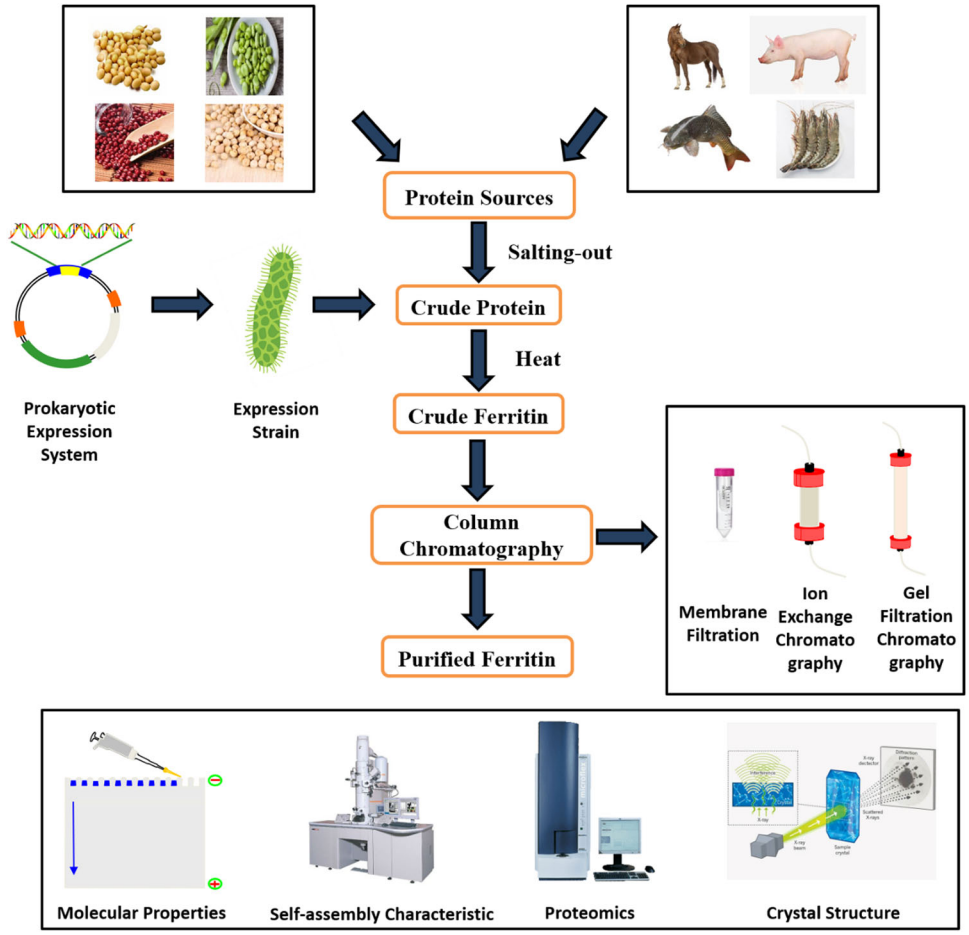

Figure 3. Isolation, purification and characterization of ferritin. Ferritin is widely found in animal internal organs and legumes, and it can also be obtained by heterogeneous expression. Ferritin can be purified by heating, salting out and column chromatography, and identified by electrophoresis, transmission electron microscopy, MALDI-TOF and crystal diffraction.

composed of a subunit. Zheng et al. successfully cloned the ferritin subunit of flatfish, and proved that ferritin has the effect of regulating immune and the activity of antibacterial in fish (Zheng et al. 2010). Human ferritin recombinant L-chain was expressed by prokaryotic expression, however, it will form inclusion bodies in *E coli*, resulting in low yield as low as 2–5 mg/mL. Molecular chaperones are proteins that assist in the assembly of molecules and in the folding of proteins. With the rapid development of genetic engineering and metabolic regulation, introducing foreign genes into *E. coli* for expression may improve the benefits of genetic engineering. Zou et al. increased the expression of the soluble part by adding molecular chaperones, thus increasing the content of ferritin (Zou et al. 2016). The experimental advances laid a foundation for the isolation and purification of ferritin and its subsequent structural characterization.

The extraction of high purity ferritin is the key to study the molecular structure of ferritin. For plants with high content of ferritin can be obtained by traditional centrifugal, co-precipitation and column chromatogram. However, for animals, which only exist in organs with high metabolic rates, it is difficult to obtain purified protein only by the commonly used gel filtration chromatography and ion exchange chromatography, while affinity chromatography and high performance liquid chromatography (HPLC) are difficult to be popularized in all laboratories. The construction of vector for heterologous expression for the purification of micro ferritin or large amount of ferritin, with low cost, fast speed and high recovery rate, which is an effective method for the purification of high purity ferritin.

Identification of ferritin

With the further development of ferritin isolation and purification techniques, the identification methods have gradually become the focus of research. Electrophoresis, electron microscopy and spectrometer methods are the most widely used method for ferritin identification. With the continuous advance of quantitative proteomics and the development of experimental techniques, studies such as peptide mass fingerprinting and crystal structure analysis of ferritin are also emerging. The flow diagram of ferritin isolation and identification is shown in Figure 3.

Molecular characteristics

Ferritin is composed of 24 subunits, the relative molecular weight of ferritin can be analyzed by electrophoresis (SDS-PAGE and Native PAGE). During the course of sample preparation and electrophoresis, ferritin molecules will

depolymerize to polypeptide chains due to the addition of protein denaturants. The molecular weight of ferritin was determined by electrophoretic mobility, therefore, ferritin can be identified according to its subunit molecular weight. In the process of sample preparation and experiment of Native-PAGE, no denaturant added, no heating treatment, so ferritin will maintain its physiological activity. Ferritin, a protein rich in α-helix, which is primarily maintained by hydrogen bonds and electrostatic interactions, and the secondary structure of different sources of ferritin is very similar. Fluorescence spectra of proteins are mainly affected by aromatic amino acid residues (tryptophan, tyrosine and phenylalanine). Because the side chains of aromatic amino acid residues have significantly different color rendering property, different fluorescence spectra will be generated, of which tryptophan has the highest fluorescence intensity (Liu et al. 2010). It was found that tryptophan existed at the end of E helix. With the electron transition of tryptophan, the fluorescence value of tryptophan increased. Therefore, fluorescence spectra could be used to characterize the structural changes of ferritin.

Deng et al. isolated and purified natural soybean ferritin. SDS-PAGE showed that soybean ferritin was composed of two subunits, 28.0 kDa (H-1) and 26.5 kDa (H-2). The ratio of the two subunits was about 1:1, and the result of Native PAGE showed that the molecular weight of purified soybean ferritin was about 560 kDa (Deng et al. 2010b). The structure of ferritin is similar to that of previously reported ferritin, which is composed of 24 subunits.

Self-assembly characteristics

The ultrastructure of micron dimension can be observed by transmission electron microscopy (TEM), and the resolution of TEM is about 0.2 nm. Ferritin has a spherical structure with an outer diameter of 12 nm and an inner diameter of 8 nm. Ferritin can be negatively stained with uranyl acetate or phosphotungstic acid, and the staining solution enters into the cage of ferritin, making it present a unique structure under transmission electron microscope. The size distribution of ferritin is ∼12 nm and the ferritin solution is a monodisperse suspension, the molecular size and particle size distribution of ferritin can be identified by dynamic light scattering (DLS). At the same time, it can also be used to monitor the variation trend of ferritin particle size to show the trend of ferritin aggregation or depolymerization. DLS can be used also to analyze the stability of ferritin and the change of molecular structure under pH and temperature variations.

Li et al. used TEM to observe the appearance and morphology of ferritin under different thermal treatment conditions. The results showed that the untreated oyster-derived ferritin, after being negatively stained by uranyl acetate, presented a monodisperse hollow spherical structure of about 12 nm. At the same time, as the temperature rises, aggregation occurs at 90 °C and 100 °C (Li et al. 2020). TEM can directly indicate whether ferritin has hollow structure, so it is the most common and simple method to detect the morphology of ferritin. Zou et al. purified the recombinant human heavy chain ferritin. The size distribution of ferritin was measured by DLS, and the particle sizes of recombinant human heavy chain ferritin and soybean ferritin were proved to be 7.20 ± 0.83 nm and 11.44 ± 3.23 nm, respectively (Zou et al. 2016). The above experiments also proved that the particle size of ferritin without any treatment had a certain difference, but it still had good monodispersity.

Peptide mass fingerprinting

According to the rapid development of proteomics study, the use of peptide mass fingerprinting has become a rapid and accurate identification method. Firstly, ferritin was digested into different peptides by the corresponding protease hydrolysis and trypsin is commonly used protease. The molecular weight of the peptides was the unique peptide mass fingerprint of ferritin. Ferritin could be identified by comparing the above fingerprints with the known protein fingerprints in the databases at National Center for Biotechnology Information (NCBI). At the same time, ferritin derived from different materials can be matched to determine whether it comes from the same precursor.

The black beans and soybean ferritin were analyzed by electrophoresis by Deng et al, and the band of 28.0 kDa and 26.5 kDa was selected for In-gel digestion. MALDI-TOF-MS/MS was used to identify the samples after enzymatic hydrolysis and the results were identified using BLAST analysis with genome sequence from NCBI. The results of charge mass ratio in mass spectrometry showed that the 26.5 kDa subunits of ferritin from black beans and soybean had high homology of amino acid sequence. At the same time, the experiment showed the significant differences in the peptide mass fingerprints of the two ferritin subunits, indicating that the two subunits were derived from different precursors, indicating that ferritin in legume may be encoded by multiple genes (Deng et al. 2010a).

Crystal structure

With the further development of physics and chemistry analyze methods, the advanced structures of proteins can be observed through the crystal structure of proteins, and the secondary structure of proteins can also be inferred. However, it is often used for the final identification of ferritin due to the time-consuming crystallization process and the need for conditional screening in crystal growth system. Ferritin is usually crystallized by the method of vapor diffusion in hanging drops. The crystal nuclei of ferritin were formed by forming supersaturated solution in a confined space and with the help of screening reagent. The crystal structure was obtained by X-ray diffraction and data analysis. At present, the initial structure of ferritins was obtained by CCP4 software. Then the structures were optimized using PHENIX software, and the COOT software was refined to obtain the crystal structure of ferritin.

Taking human recombinant L-chain ferritin as raw material, Zhang et al. cultured the crystal and analyzed the

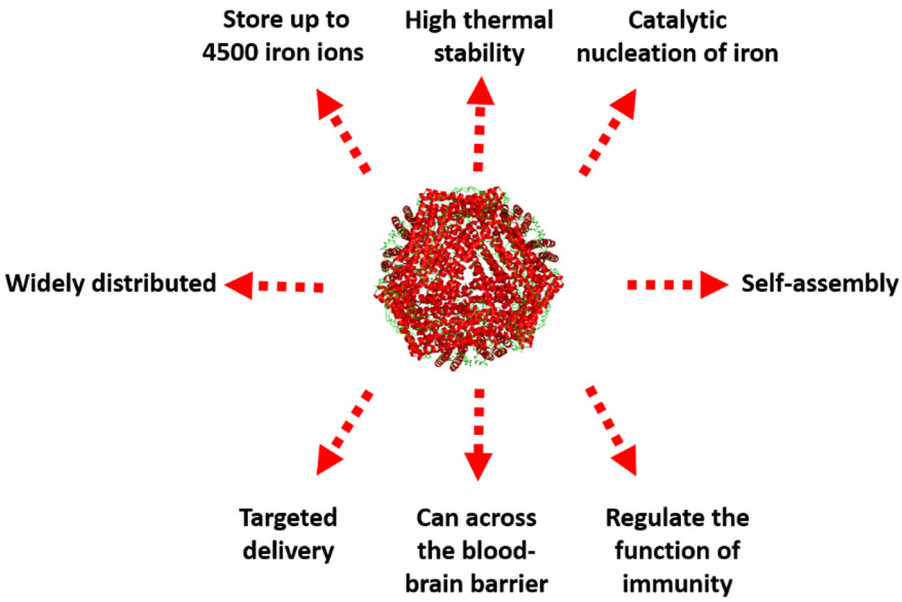

Figure 4. Function of ferritin. Ferritin is distributed widely in the nature and has some functional properties due to its hollow structure and catalytic activity.

crystal structure, and the major forces affecting the subunit self-assembly and the surface tension of the structure was analyzed. Ferritin mutants with different number and shape of subunit, different oxidation ability and cancer cell recognition ability were constructed by gene mutation. The crystal structure of the mutants was further analyzed to explain the causes of physical and chemical changes at the atomic level and microstructure (Zhang et al. 2017). The analysis of the structure can provide a theoretical basis for the study of modification and function, and then affect the self-assembly of ferritin. These manipulations may improve the activity of ferritin, which plays an important role in following research.

Function of ferritin

Regulates the balance of iron metabolism

Due to the unique structure and physiological activity of ferritin, ferritin has many functional properties, as shown in Figure 4. Iron is one of the essential nutrient elements in the body and plays an important role in specific biochemical reactions. Iron is the raw material in the synthesis of hemoglobin and myoglobin. Meanwhile, it can participate in the oxidation-reduction reaction in vivo as a coenzyme, and it plays an essential role in oxygen transport, electron transport and catalysis (Andrews 2008). Lack of iron in the body can affect the normal physiological reactions, and then lead to the corresponding disease. In recent years, iron deficiency anemia (IDA) has become a devastating disease threatening human health, especially the consequences of cardiovascular and neurological disorders. When the content of iron in the body increased significantly, ferritin can catalyze Fe^{2+} to Fe^{3+} and mineralize to iron core for storage (Lawen and Lane 2013). But when the body suffers from iron deficiency, the iron ions can be released by ferritin for use. At present, there are two main speculations about the iron release mechanism of ferritin: the release of iron through lysosomes or directly (Balla et al. 1992; Huang and Barker 1983).

Antioxidative properties of ferritin

Free Fe^{2+} in the human body has high toxicity. Under aerobic conditions, the reaction of Fe^{2+} with H_2O_2 produces hydroxyl radicals, which has powerful oxidization and called Fenton reaction (Cadenas 1989). Studies have shown that highly reactive free radicals can accelerate protein oxidation and the modification of nucleic acid. Reactive nitrogen damages proteins through nitridation reaction, and oxidative stress occurs when the content of reactive oxygen species is higher than the antioxidant capacity of the body. Excess iron will accelerate the occurrence of oxidative stress, thus the pathological phenomena will be aggravated (Cozzi et al. 2000). Excessive accumulation of ferritin in plants will reduce the concentration of free iron ions and the possibility of antioxidant stress (Chen and Barak 1982).

Participates in immune response

Ferritin can play a role in the innate immune system of higher vertebrates (Rogers et al. 1990). Studies have shown that the ferritin expression of turbot can be significantly increased under the stimulation of *Streptococcus* and *Edwardsiellosis* (Zheng et al. 2010). The stimulation of *Vibrio anguillarum* can also increase the expression content of ferritin in the internal organs of large yellow croaker (Zhang et al. 2010). Adding ferritin to white shrimp can significantly enhance immune response (Ruan et al. 2010). The ferritin content of horseshoe crabs infected with the bacteria also increased significantly (Ong et al. 2005). High concentration of iron ions and bacterial stimulation can increase the expression content of ferritin in scallops (Jin et al. 2011). The above studies have proved that ferritin can participate in the innate immune response of the body. In vitro, the bacteriostatic effect of ferritin was also demonstrated. Ferritin can restrain the growth of pathogenic bacteria in turbot and *Vibrio anguillarum* in scallop (Zhang et al. 2013). There is a similar immune response in plants as the

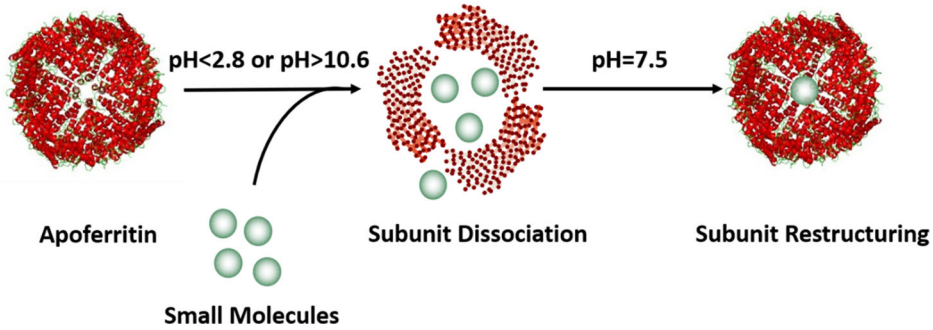

Figure 5. The self-assembly characteristic of ferritin regulated by pH. Regulating the pH of ferritin can regulate the depolymerization and self-assembly of the ferritin subunits, then the embedding and the targeted transport of the material can be carried out.

accumulation of ferritin can affect fungal or viral infections, thereby protecting cells from damage (Liu, Chen, and Chou 2003).

Application of ferritin

Applications as nanocarriers

Ferritin is a natural storage protein, which can be self-degraded and has high biocompatibility, causing no allergic reactions. Ferritin can embed substances via free diffusion with a diameter smaller than the ferritin channel since the surface of ferritin has 8 hydrophilic channels. It can be used for tagging with fluorescence reporter gene or fluorescent dyes and transporting hydrophilic drugs and some metal ions, such as Mn, Cd, Au, Co, etc (Douglas and Stark 2000). The 6 hydrophobic channels can be used to deliver hydrophilic drugs or to load imaging contrast agents. Therefore, as a protein carrier, ferritin can encapsulate both hydrophilic and hydrophobic substances. After obtaining apoferritin, small molecules can enter and bind to the ferritin cavity by means of free diffusion. Due to the presence of non-covalent binding in ferritin, the method is simple, with high selectivity for the embedding material. Ferritin has often been used to bind materials containing metal cores, and the commonly used methods are: 1. Nucleation of mineralization (Yamashita, Iwahori, and Kumagai 2010). 2. Genetic modification to let ferritin has the ability to bind materials (Kramer et al. 2004). 3. Reduce the material to metal and embed (Ueno et al. 2004; Douglas and Stark 2000). At the same time, materials with a diameter larger than that of the ferritin channel can also be embedded. It was demonstrated that 24 subunits were bonded by hydrogen bonding, salt bridging or hydrophobic interaction (Kim, Ahn, et al. 2011a). As shown in Figure 5, ferritin can be depolymerized into 24 subunits under extreme conditions (pH $>$ 2.8 or pH $<$ 10.6) or in the presence of protein denaturation agents, and the commonly used denaturing agents such as carbamide. Similarly, when solvent environment returns to neutral, ferritin will self-aggregate into nanocage structures (Stefanini, Vecchini, and Chiancone 1987). Thus, ferritin can be turned into a hollow structure by the release of the iron core, and the cavity of ferritin can be used to load other substances, such as water-soluble substances. Ferritin can be used as a drug carrier due to its unique structure and function and has important applications in medicine (Yang et al. 2007).

Transferrin receptor (TfR) is one of the most representative receptors, which exists on the surface of many cells and mediates the entry of iron-containing ferritin from the extracellular to the intracellular (Huang et al. 2019). TfR is often over-expressed in cancer cells. Ferritin nanomedicines can successfully cross the blood-brain barrier and bind to TfR, thus providing targeted therapy for some malignant tumors (Li et al. 2010). In the study of Fan et al., ferritin can bind to TfR and cannot be degraded by lysosomes under endocytosis. Then it can cross the blood-brain barrier and enter glioma cells, enriched in lysosomes and then degraded, released the drug loading in the ferritin. Therefore, ferritin has become a unique method for the treatment of central nervous system diseases and has great potential (Fan et al. 2018).

Compared with the known drug delivery methods, the use of ferritin as a drug delivery carrier has the following advantages: (1) increased drug absorption for drugs insoluble in water or poorly soluble in water; (2) the efficacy of the drug at the action sites can be monitored; (3) can be used with multiple medications; (4) the reaction can take place at a specific location; (5) through selective action, the drug concentration at the place of administration can be increased and the degradation of the drug can be reduced.

Applications in tumor spread monitoring

Excess iron can cause cancer, and free iron can cause damage through the Fenton reaction, which can lead to cancer. The content of serum ferritin was found to be correlated with the occurrence of cancer. Under normal physiological conditions, the content of ferritin in serum tends to be stable. In pregnancy and anemia, the content of ferritin decreased, but in the special site where tumor grown, the content of ferritin increased significantly. Oncoembryonic ferritin is a special ferritin in liver cancer, which is often used in the diagnosis of liver cancer. Recent studies have shown that in breast cancer (Orlandi et al. 2014), lung cancer (Kukulj et al. 2010) and pancreatic cancer (Kalousová et al. 2012), the content of iron ion in the body of the patients is much higher than that of the normal body, so the ferritin content will also increase significantly. Therefore,

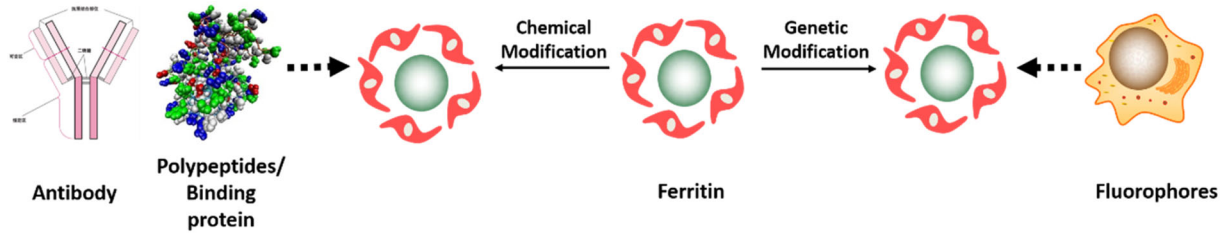

Figure 6. Modification of ferritin molecules. Through chemical modification and genetic modification, ferritin can be connected with functional substances, and ferritin can have the characteristics of functional substances, so as to achieve the purpose of molecular modification.

ferritin can be widely used in the fields of monitoring of cancer (Fan et al. 2012).

Applications of ferritin after modification

Due to the unique physiological activity of ferritin, molecular modification of ferritin has become a research hotspot, mainly including chemical and genetic engineering methods to modify the function of ferritin. Figure 6 shows the methods of binding functional substances to ferritin. Chemical modification needs to meet stricter environmental conditions, such as pH and temperature, which may affect the activity of functional substances (Danon et al. 1972), so the use of genetic engineering to modify ferritin gradually attracted attention. Genetic modification is to directly change the gene of ferritin and bind the required material sequence to the end of the ferritin sequence, using experiments of recombination and expression to achieve the purpose of modification. The conditions for this modification are relatively simple, easy to implement and regulate, steady performance which would not be influenced by the environment. The introduction of exogenous gene in the process of experiment does not have toxic effects. At the same time, the expression and folding of ferritin will not be affected during this process. The modified ferritin will have the properties of functional substances, ferritin can therefore be connected to a drug or nutrient, providing technical support for the development of novel proteins and drugs (Wang, Mercogliano, and Löwe 2011). By genetic modification, Ueno et al. made ferritin have a site for binding specific metal ions. Transporting metal ions into cells and can release carbon monoxide under certain conditions, thereby modulating cytokines (Fujita et al. 2014, 2016). Matsui et al. linked the N-terminus of ferritin with hydrophobic peptides by genetic modification. The modified ferritin can be attached to the carbon of simple substance phase, thus affecting the assembly of ferritin (Matsui et al. 2007). Kim et al. linked the green fluorescent protein (GFP) to the surface of ferritin via peptides, making the modified ferritin easier to modify (Kim et al. 2011b).

Summary

Ferritin is a protein cage composed of the same 24 subunits symmetrically, and it has the characteristics of high temperature resistance and extreme pH resistance. It can be dissociated into a single subunit under certain conditions and self-assembled into a complete 24 polymers in a neutral environment. In this review, the distribution and structural characterization of ferritin from different sources were presented. The isolation and purification of ferritin from plant and animal sources were discussed, and the application of prokaryotic expression to obtain ferritin based on the development of molecular biology was also demonstrated. In this paper, various methods for identifying ferritin are introduced, such as molecular, self-assembly and structural properties, and the applications of ferritin were reviewed. Studies have shown that ferritin can be widely used in the development of iron supplements, targeted drugs, nutrient delivery, tumor monitoring and other fields, but the mechanism of action of ferritin in the above applications and the relationship between structure and function remain to be further studied.

Ferritin can be obtained through traditional isolation and purification, but due to the complex procedures and low yield, as well as the development of genetic engineering, prokaryotic expression has gradually attracted public attention. Meanwhile, adding molecular chaperones and increasing conditional screening will greatly improve the yield and purity of ferritin. The structure of ferritin determines its properties, such as embedding, transports nourishing compounds and the use as iron-supplementary, etc. Therefore, ferritin can be modified by structural analysis, and through gene mutation and molecule modification, a new type of ferritin with unique properties can be constructed, so as to expand its application and economic value.

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