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Purification, characterization, protein association property of a non-muscular actin from porcine lung

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Abstract

Global pork production was 101 million tons in 2020. Consequently, a large amount of the porcine viscera were produced simultaneously, of which porcine lung is one major component, so the utilization of these residual materials has garnered significant attention in recent times. In this work, a non-muscular actin (APL) was successfully isolated and purified to homogeneity from porcine lung tissue using a straightforward and efficient protocol. Approximately 100 mg of the protein was obtained from 1 kg of porcine lung tissue. The experimental results showed that the native APL exhibits an apparent molecular mass of about 42 kDa. The amino acid composition analysis demonstrated the presence of all eight essential amino acids in APL. More importantly, APL is rich in magnesium and calcium ions (approximately 33 magnesium ions and 18 calcium ions per molecule). These two metal ions have a great effect on the structure and function of APL. As far as we are aware, this work provides the first systematic report on the purification and characterization of non-muscular actin from porcine lung which represents a newly potential class of magnesium and calcium supplement.

Keywords Porcine lung, magnesium ions, calcium, actin, polymerization

1. Introduction

During the last 60 years, global pork production increased from 24.7 million tons in 1961 to 115.5 million tons in 2023 [1]. According to the statistics data of American department of agriculture global pork production was around 107 million tons per year in the recent five years [2]. Consequently, thousands of tons of the porcine viscera were produced simultaneously, which were regarded as wastes. Porcine lung was one major component in the visceral wastes. These waste materials present significant environmental challenges, including disposal difficulties, potential pollution risks, and the loss of valuable biomass and essential nutrients. The bioconversion of food processing residues has attracted growing attention due to their potential as valuable resources for transformation into useful products. To sum up, the utilization of meat processing waste as a source of functional ingredients is a promising field of research.

Actins, a family of evolutionarily conserved proteins ubiquitous in eukaryotic organisms, serve critical functions in both muscle contraction and diverse cellular activities within non-muscle cells. These versatile proteins exhibit dynamic structural polymorphism, alternating between monomeric globular subunits (G-actin) and polymeric filamentous assemblies (F-actin). This structural duality enables actins to fulfill their essential roles in cellular motility, cytokinesis, and intracellular transport mechanisms [3-5]. When the concentration of G-actin exceeds a critical threshold, it undergoes spontaneous polymerization to form F-actin filaments, which play essential roles in maintaining cell shape, facilitating cell motility, and supporting various other cellular functions. Actins are classified as α, β, or γ in order of increasing basicity that results from variation among the acidic residues at the N-terminus while different tissues display differences among these types [3, 5]. Muscle actin is a subtype of the actin family that is specifically involved in muscle contraction, mainly including α-skeletal muscle actin, α-smooth muscle actin and α-myocardial actin. These subtypes are highly conserved in muscle cells, and their main function is to interact with myosin to form myofibrils, thereby causing muscle contraction and relaxation. Non-muscular actin is a type of actin family. Compared with muscular actin, it mainly exists in non-muscular cells and mainly includes two subtypes: β-actin and γ-

actin. The only difference is the four amino acids located at the N-end of the polypeptide chain, which are necessary for survival and normal cell functions.

Non-muscular actin is the main component of the actin cytoskeleton, and the actin cytoskeleton is an important system involving various aspects of cellular function, including the regulation of cellular motility, structure, integrity, signal transduction and transcription [6]. In addition to its essential roles as part of the cytoskeleton, actin has also been linked to many processes in the nucleus. Recent data has demonstrated the presence of both monomeric and polymeric actin in the nucleus, and implied distinct functional roles for these actin pools [7]. Monomeric actin seems to be involved in regulation of gene expression through transcription factors, chromatin regulating complexes and RNA polymerases. Besides of structural roles, nuclear actin filaments may be required for organizing the nuclear contents and for the maintenance of genomic integrity.

Actin exists in both a monomeric globular state (G-actin) and polymerized filamentous state (F-actin) [8]. G-actin, with a molecular weight of 42 kDa, is composed of a single polypeptide chain that contains approximately 375 amino acids. The actin monomer consists of two primary domains, with each domain further subdivided into two distinct subdomains. Actin generally contains one molecule of ATP (or ADP) and one divalent cation (Mg^{2+}) as cofactors. In vitro, Mg^{2+} is usually replaced by Ca^{2+} [9, 10]. These cofactors are positioned within the interdomain cleft formed by the two structural domains. Removal of calcium ion by a chelator results in the transformation of G-actin into the inactivated form, in which the protein molecule loses its capability to polymerize [11, 12]. These observations demonstrate that the divalent ion such as Mg^{2+} or Ca^{2+} is required for actins to maintain their function.

Actin has good application potential. In terms of food safety monitoring, actin can be used for the detection of meat adulteration [13], toxin detection [14], and microbial contamination monitoring [15]; In terms of hydrogel construction, its self-assembly property can be used to deliver bioactive substances (such as probiotics, nanoparticles); In the field of nanotechnology applications, actin-myosin systems are used to construct nanoscale biosensors or laboratory chip devices [16].

At present, there are relevant studies on extracting actin from pigs. A simultaneous actomyosin and actin isolation procedure from post-rigor porcine muscle was developed, based on differential solubility, gel filtration chromatography and extraction steps. The isolated actin preserves its polymerisation ability [17]. Nakamura and Migita et al [18] examined the effects of nucleoside monophosphates on the dissociation of actomyosin into myosin and actin. They found that IMP enhances the extraction of myosin and actin from porcine meat. So far, there have been no relevant studies on extracting actin from pig lungs.

During this research, we successfully isolated and purified a non-muscular actin (APL) to homogeneity from porcine lung tissue for the first time. Interestingly, this actin naturally binds magnesium and calcium ions with high capacity (approximately 33 magnesium ions and 18 calcium ions per protein molecule), while the content of other minerals for instance, zinc, iron, and copper is much lower. These magnesium and calcium ions have a significant impact on the structure and ability of APL. On the other hand, APL is rich in all essential amino acids, the content of which is higher than that in soybean protein isolate except for phenylalanine. Thus, this new protein could represent a new class of materials from natural sources with a great potential for magnesium and calcium supplements.

2. Materials and methods

2.1 Chemicals

N′,N′-bis-methyleneacrylamide, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), Tris (hydroxymethyl) aminomethane, TEMED, β-Mercaptoethanol, Coomassie brilliant blue R250, diethyldithiocarbamate (DEDTC) were obtained from Sigma-Aldrich Co. (Beijing, China). The native electrophoresis marker and SDS electrophoresis marker were purchased from GE Healthcare Bio-Sciences AB (Beijing, China). All reagents utilized in the experiments met or exceeded analytical grade standards.

2.2 Protein purification

APL was purified by the standard procedure with some change [19]. Fresh porcine pulmonary obtained from the local market were cleaned, grinded by automatic meat grinder at 4°C and stored at -80°C reserved for later. Typically, approximately 250 g of the grinded porcine pulmonary was soaked in 150 mL 100% cold acetone, and centrifuged at 10000 × g, 4°C for 15 min. Then the precipitate was soaked in 250 mL buffer A (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP), and mixed round for nearly 3-4 h at 4°C, centrifuged at 10000 × g, 4°C for 20 min to separate the insoluble material. Then mixed the supernatant with buffer B (2 mM Tris-HCl, pH 8.0, 1.0 mM ATP, 2 mM $CaCl_2$, 5 mM KCl), when the mixture became turbid and ropy, adding dropwise the KCl to whose terminate concentration was 0.6 M. Then the mixture was centrifuged at 12 000 × g, 4°C for 20 min to get the precipitate, which was full of actin. Then the precipitate was re-suspended with 2 mM Tris-HCl buffer without ATP. The re-suspended precipitate was dialyzed against four changes of the buffer A at 4°C for 24 h, centrifuged at 12000 × g, 4°C for 20 min to separate the insoluble material. The supernatant was collected and subjected to an ion-exchange column (DEAE, Solarbio), followed by gradient elution with 0–0.5 M NaCl. After further purification by a gel filtration column (Superdex 200, GE Healthcare), equilibrated with 50 mM Tris-HCl and 150 mM NaCl (pH 8.0), the purity of resultant protein was verified by SDS-

polyacrylamide gel electrophoresis (SDS-PAGE), the protein was concentrated and stored at 4°C for later use. Protein concentrations were determined according to the Lowry method with BSA as standard.

2.3 In-gel trypsin digestion and peptide mass fingerprinting (PMF) by MALDI-TOF-MS

The electrophoretic separation of APL was performed on a 15% SDS polyacrylamide gel. Following electrophoresis, two distinct bands measuring 1 mm in width were carefully excised using a pipette tip and subsequently transferred into 1.5 mL microcentrifuge tubes. Each tube received 100 μL of destaining solution containing 50 mmol/L NH_4HCO_3 in 50% acetonitrile (ACN), followed by a 20-minute incubation period. The solution was decanted, this washing procedure was repeated for 3 cycles or until complete disappearance of the blue coloration from the gel fragments was achieved. After that 200 μL of 100% ACN were added and incubated for 10 min. The gel fragments transitioned from a semi-transparent to an opaque white appearance. Following this change, the solution was decanted, and the gel pieces were subjected to vacuum drying in a SpeedVac for 15 minutes. Then 5 μL of trypsin solution (0.005 μg/μL in 25 mmol/L NH_4HCO_3, pH 8.0) were added to each tube and incubated at 37°C over night. The gel pieces were treated with 50 μL of an extraction solution containing 50% ACN and 0.1% TFA for 30 minutes to recover the peptides. After extraction, the peptide solution was reduced to about 10 μL using a SpeedVac and then kept at -20°C for later use. Before MS analysis, the peptides were cleaned and concentrated using Ziptip C-18 columns.

Peptide mass fingerprinting (PMF) was detected by MALDI-TOF-MS., and MALDI-TOF-MS analysis was conducted using a Bruker ultraflex III instrument (Germany) operated in positive ion mode. The instrument settings included a 20 kV acceleration voltage and a 337 nm nitrogen laser. For internal calibration, trypsin's self-digestion products were used as reference standards. Prior to analysis, APL peptide samples underwent desalting through spin column purification (Pierce brand).

For sample preparation, 0.5 μL of peptide solution was mixed with an equal volume of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in 50% ACN/0.1% TFA at 10 mg/mL) on a stainless steel target plate, followed by natural air-drying. During data acquisition, the laser was systematically scanned across the sample surface, delivering approximately 100 pulses grouped in 10-shot clusters. All spectral data were collected in positive ion reflection mode.

The acquired peptide mass fingerprints were analyzed through the MASCOT database search platform (www.matrixscience.com) against SWISS-PROT and NCBInr databases, allowing for one potential missed cleavage site during the search process.

2.4 Analysis of amino acid composition of APL

The amino acid composition of APL was determined using a Hitachi L-8900 Amino Acid Analyzer. In short, the process included hydrolyzing the sample with 6 M HCl at 110°C for 24 hours in a sealed tube, followed by eluting the hydrolysate with a series of sodium citrate buffers ranging from 0.2 M, pH 3.25, to 0.35 M, pH 5.25. Each sample required a total analysis time of 2.5 hours. Tryptophan content was not measured.

2.5. Analyses of metal ions in protein

The analysis of different metals present in APL was carried out through inductively coupled plasma optical emission spectroscopy (ICP-OES), employing a Thermo Scientific iCAP 6000 Series ICP instrument from Thermo (http://www.thermofisher.cn/).

2.6 Removal of metal ions from APL

To prepare apo APL, the native APL was subjected to extensive dialysis through four changes of 50 mM Tris-HCl buffer (pH 7.2) containing 5 mM EDTA (1.0 L each) at 4°C. Subsequently, the solution was dialyzed against EDTA-free buffer under the same conditions for 24-36 hours, with four buffer changes, to ensure complete removal of unreacted EDTA. The final protein preparation, with a concentration of 9.87 μM, was stored at 4°C for subsequent use.

2.7 Circular dichroism (CD) and fluorescence spectroscopy

CD spectral measurements were conducted on a PiStar-180 spectrometer (Applied Photophysics) at 25 ± 1°C with nitrogen gas purging. Samples containing either EDTA-treated or native APL (1.5 μM in 2 mM Tris-HCl, pH 8.0) were thermally equilibrated before analysis. Data collection spanned the 190-260 nm range, with each spectrum derived from quadruplicate scans followed by buffer reference subtraction. Protein secondary structure parameters were computationally analyzed using CDNN 2.1.0 software based on the acquired far-UV CD profiles. Continuous nitrogen flow was maintained during measurements to prevent atmospheric interference. Fluorescence measurements were carried out with a Cary Eclipse spectrofluorimeter (Varian). At 25°C, excitation-emission spectra were collected, and emission spectra (λem: 310-550 nm) were obtained via data processing at the maximum excitation wavelength of 295 nm. All experiments were conducted in triplicate.

2.8 Polymerization property of APL

APL polymerization was monitored by light scattering measurements using a Cary Eclipse spectrofluorimeter (Varian) as previously described [20]. 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. Uranyl acetate was used to stain the samples.

2.9 Structure prediction of APL

The sequence information was blasted and acquired on National Center for Biotechnology Information (NCBI databank). The trRosetta program was used to predict the tertiary structure of APL. Pymol software was used to analyze the possible binding sites of Mg^{2+} and Ca^{2+} in the predicted structure of APL.

2.10 Statistical analysis

The data were analysed by Statistical Analysis System (SAS 9.0; SAS Institute Inc., Cary, NC) package software for analysis of variance and Duncan's test. All experiments were carried out in triplicate.

3. Results and discussion

3.1 Isolation and characterization of actin from porcine lung

There were billions of alveolar cell on porcine lung, and actin was plentiful as the composition of the cytoskeleton. Our research focuses on whether or not porcine lung is rich in actin. If so, what character it is. To answer this question, actin from porcine lung was isolated and purified. The procedure is mainly composed of two major steps: formation of "acetone powder", and preparation of F-actin. Non-denaturing gel electrophoresis (native PAGE) resolved purified protein as a single complex (Figure 1A), indicating that the protein is purified to homogeneity. Following treatment of the purified protein sample with β-mercaptoethanol, the SDS-PAGE results similarly displayed a single band (Figure 1B, lane 1) with an approximate molecular weight (MW) of 42 kDa. These findings suggest that the purified protein exists as a monomer in its native state, consisting of one subunit. Its MW is the same as reported actins [3, 4, 9, 12]. According to the above procedures, ~100 mg of actin was yielded from 1 kg of porcine lung. This yield is relatively high, indicating that the porcine lung is rich in non-muscular actin.

To obtain more information about this protein, the peptide mass fingerprinting (PMF) of the SDS-PAGE gel band of 42-kDa were obtained by MALDI-TOF mass spectrometry using CHCA as the matrix, respectively. The MALDI-TOF-MS spectrum of the gel band 42-kDa generated from in-gel trypsin digestion is shown in Figure 2A. Concurrently, the MALDI-TOF spectrum of a control background-only band was also acquired, using the same in-gel trypsin digestion method. The PMF of gel band "42-kDa" is summarized after excluding the trypsin autolysis products from the control spectrum. It had three abundant peptide ions of m/z 1791, 1515 and 3489. Figure 2A also reveals some relatively weak peaks, including m/z 800, 1199, and 2279. Subsequently, a MASCOT database search was conducted to gather information on the best - matching protein for APL. The peptide mass data of the 42 - kDa gel band was input into the MASCOT search program (www.matrixscience.com) for a search in the protein database NCBInr 20110911, which comprises 15,270,974 sequence entries. The PMF of the 42-kDa subunit (Figure 2A) matches up to 100% with that of non-muscular actin from the porcine tissures (*Sus scrofa*, [21]), suggesting that APL is a kind of non-muscular actin.

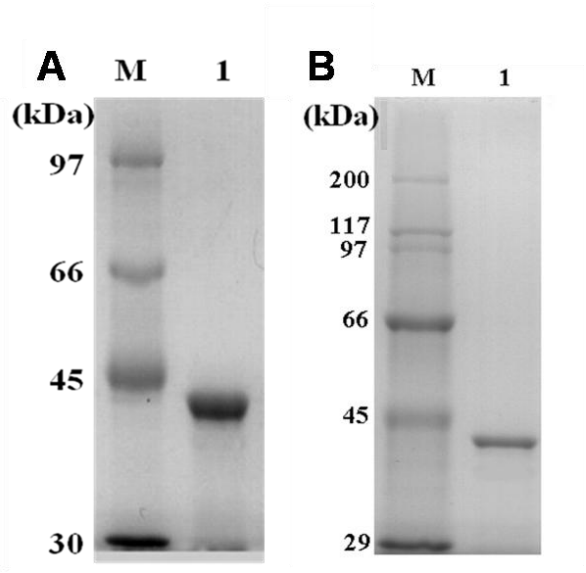

Figure 1 Native PAGE and SDS-PAGE analyses of purified actin from porcine lung. (A) Native PAGE. (B) SDS-PAGE. *Lame M*, protein markers and their corresponding molecular masses; *Lane 1*, AGO.

Moreover, six amino acid sequences were acquired from MALDI-TOF-MS (Figure 2B). These six peptide fragments were checked against the non-redundant (nr) protein sequence database using the Blast Algorithm, which best matches actin from *Sus scrofa*, confirming the above conclusion. In our previous report, we isolated the actin from oyster, which shows typical metal binding capacity [22]. Comparably, actins from different species shared highly conserved amino acids sequence and tertiary structure. This may be caused that actins play important roles as the composition of muscle. But based on the various living environment of different species, their actins were endowed with specific characteristics. Thus we are curious if actin from porcine lung has any unique features, and the reason.

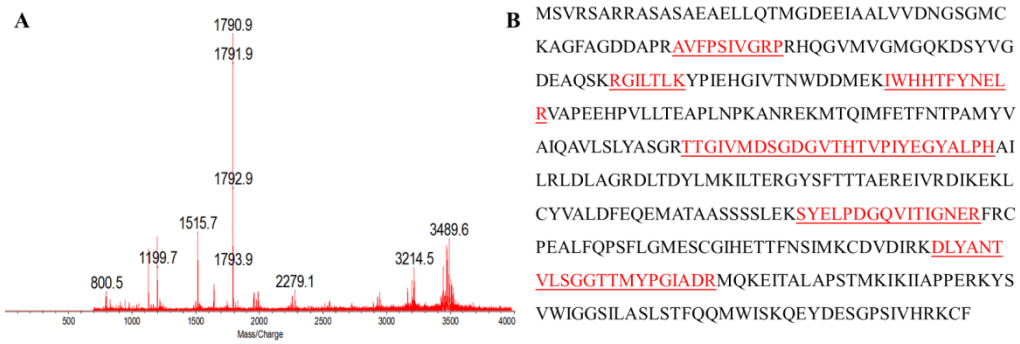

Figure 2 (A) Tryptic PMF of APL gel band from SDS-PAGE acquired by MALDI-TOF-MS. (B) Peptide sequences of APL acquired by MALDI-TOF-MS.

3.2 Analysis of amino acid composition of APL

Soybean protein isolate (SPI) is a plant-based complete protein with a rich and balanced amino acid composition, which makes it an ideal control sample for comparing the amino acid composition of other proteins [23]. The amino acid distribution of SPI is balanced, providing all essential amino acids and most non-essential amino acids. This enables it to provide reference standards similar to human body needs in amino acid composition analysis.

To analyze the amino acid composition, APL was hydrolyzed with 6 M HCl for 24 h at 110°C in a sealed tube with SPI as a control sample, followed by analyses of different amino acid contents, and results were summarized in Table 1. APL contains all kinds of amino acids which are required for protein synthesis, and many amino acids are plentiful besides aspartic and glutamic acids. More importantly, APL is rich in all essential amino acids (EAAs). Numerous studies indicate that essential amino acids (EAAs) alone are sufficient to stimulate muscle protein synthesis, suggesting that proteins rich in these amino acids are highly effective in supporting muscle growth [24, 25]. It is known that soy bean protein-based formulas have been widely used as the source of nutrition for infants due to their good amino acid composition [26]. Interestingly, the EAAs content of APL in total amino acids is 52.19 (g/100 g protein), which is higher than that of SPI (34.35 g/100 g protein). Especially, the contents of threonine and methionine in APL are 8.22 and 6.39 g/100 g protein, respectively, and they are much higher than those in SPI (3.5 g/100 g protein for Thr and 1.3/100 g protein for Met). Similar threonine and methionine contents in SPI were reported from different research groups [27, 28]. Whey protein (WP), a well proportioned protein source, has also been reported to be able to enhance physical well being with EAAs supplements [29, 30]. Compared with the content of EAAs in WP (45.13 g/100 g protein) reported recently [30], the EAAs content of APL reaches 51.2, which is higher than that of WP by 7.06 (g/100 g protein). To better illustrate the characteristics of APL's amino acid composition, we further compared it with those of the FAO/WHO amino acid reference pattern set up for humans [31]. Results showed that the eight EAAs contents of APL were all higher than those of FAO/WHO pattern. According to the amino acid scores obtained from the amino acid content of APL divided the FAO/WHO content, the maximum limiting EAAs contribute to methionine, a sulfur-containing amino acid which is important metabolically to such extent that their relative requirement for maintenance is probably higher than that for human growth [32]. Threonine ranks the second, which is particularly nutritionally important, since it has been suggested to be the second rate-limiting amino acid in the maintenance requirement after methionine [33]. It may because it accounts for the largest single component of the ileal loss into the large bowel [34]. The occurrence of tryptophan in proteins is generally less than other amino acids, however, it is rich in APL of 2.0 (g/100 g protein), which is more than triple of FAO/WHO pattern; it's also nutritionally important since it is a precursor for important metabolites such as serotonin and nicotinamide. APL bears abundant contents of other EAAs like isoleucine, valine and so on. Therefore, APL appears to have a higher nutritional value due to its better amino acid composition, and it could serve as an alternative dietary supplement of amino acids.

3.3 Analyses of the content of metal ions in APL

To investigate the presence of metal ions in APL, the levels of several metals (Mg, Ca, Cu, Fe, and Zn) in the protein were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). A very high ion intensity for magnesium was observed in this purified protein. Based on three independent experiments, the magnesium content in the protein sample (9.87 μM) was 325.00 ± 0.24 μmol/L. The stoichiometry of bound magnesium per protein was calculated as approximately 33:1 based on the molecular mass of the purified protein. As a result, a single APL molecule contains approximately 33 calcium atoms. In a similar way, the calcium content in the protein sample (9.87 μM) was determined to be 180.00 ± 0.35 μmol/L, and thus the stoichiometry of bound calcium per protein was about 18/1. Interestingly, we found that APL also contains zinc ions with the stoichiometry of Zn/protein as ~1.67/1, whereas this protein is hardly bound with either Fe or

Cu naturally (Table 2). Comparable findings were observed when analyzing protein samples from three distinct protein preparations, indicating that the varying metal contents are not specific to a particular sample. These results indicate that APL exhibits the highest ability to accumulate Mg^{2+} among these five metal ions, while having the lowest to bind Zn^{2+}, Fe^{3+}, and Cu^{2+}. Except for Mg^{2+} ions, this protein can bind to Ca^{2+}, consistent with previous studies showing that Mg^{2+} or Ca^{2+} is usually used as a cofactor in actins [9, 10, 35]. It has been known that actin is rich in Asp and Glu residues, consequently, we hold the view that these acidic residues are the key factors for the binding of Mg^{2+} or Ca^{2+} to APL. From the standpoint of nutrition, APL could be explored to be a new class of calcium or magnesium supplement because APL is rich in magnesium and calcium. For example, magnesium ions account for ~2% of the total mass in APL, and calcium ions account for around 1.7% of the total. As all know, calcium and magnesium are essential elements for human nutrition, with a broad sources, including milk, fruits, vegetables and sea foods. But according to our knowledge, this is the first report that the two mineral elements are abundantly stored in porcine lung, which is always regarded as a waste in meat processing. Thus this discovery will supply broader applications for pork, increasing its commercial value.

Table 1 The amino acid content of APL by amino acid analyzer.

Amino acid	Amino acid composition (g/100 g protein)		FAO/WHO pattern	Amino acid score
	APL	SPI		
EAAs[a]				
Thr	8.2	3.5	2.3	3.6
Leu	8.8	7.5	5.9	1.5
Ile	8.5	5.0	3.0	2.8
Val	6.7	5.0	3.9	1.7
Phe	5.1	5.3	3.8	1.3
Met	6.4	1.25	1.6	4.0
Lys	6.6	5.7	4.5	1.5
Trp	2.0	1.1	0.6	3.3
Total content	52.2	34.4	25.6	2.0
NEAAs[b]				
Asp	7.0	11.7		
Ser	6.5	5.6		
Glu	8.5	20.9		
Gly	5.2	4.0		
Ala	6.6	3.7		
His	3.3	2.3		
Arg	7.5	7.7		
Pro	5.5	5.0		

[a] Essential amino acids

[b] Non-essential amino acids

Table 2 Quantitative results of five different metal ions in porcine lung actin using ICP-OES[a].

Element	Molar concentration (μmol/L)	Metal/OA (mol/mol)
Zn	16.514 ± 0.062	1.67 ± 0.02
Ca	180.00 ± 0.35	18.23 ± 0.10
Mg	325.00 ± 0.24	32.93 ± 0.09
Fe	2.678 ± 0.035	0.27 ± 0.01
Cu	3.810 ± 0.006	0.39 ± 0.01

[a] Values are the means ± standard deviations (n = 3)

3.4 Effect of metal ions on the structure and stability of APL

To study how metal ions like Mg^{2+} and Ca^{2+} influence the structure of APL, changes in the protein's secondary structure were evaluated using CD spectroscopy, both with and without metal ions (as depicted in Figure 3A). The CD spectrum of native APL shows a composition of 92.1% α-helix, 0.7% β-sheet, 8.6% β-turn, and 1.0% random coil. When metal ions are removed from the native protein using EDTA, there is a significant reduction in the estimated α - helix content to 78.7%. Along with this decrease, the random coil content rises from 1.0% to 3.9%, and the β - sheet content experiences a slight increase from 0.7% to 2.2%. These findings show that the presence of metal ions in APL stabilizes the secondary structure of the protein, being in good accord with previous results that Mg^{2+} or Ca^{2+} is used as a cofactor for actin. The present observation also agrees with our recent report showing that a different new cofactor, Zn^{2+} occurring in

non-muscular actin from the gills or mantles of oyster (AGO) *Crassostrea gigas* also plays an important role in the stabilization of protein secondary structure [22].

To examine how metal ions (Mg^{2+} and Ca^{2+}) influence the tertiary structure of APL, the intrinsic fluorescence emission of the protein was studied (Figure 3B). When excited at 295 nm, EDTA-treated APL showed an emission peak around 340 nm, suggesting that the fluorescence primarily comes from tryptophan residues. Compared to EDTA-treated APL, native APL exhibited a reduction in overall fluorescence, along with a red shift in the emission maximum to ~345 nm. This indicates that Mg^{2+} and Ca^{2+} alter the local tertiary structure near the tryptophan residues in APL. The observed change is likely due to the increased exposure of aromatic tryptophan residues to a more hydrophilic environment when Mg^{2+} and Ca^{2+} bind to the protein [36]. Therefore, Mg^{2+} and Ca^{2+} also trigger changes in the localized tertiary structure of the protein. These findings align with previous studies on other types of actin, which demonstrate that Mg^{2+} or Ca^{2+} can modify protein tertiary structure [20].

3.5 Effect of metal ions on polymerization activity of APL

Laser light scattering is commonly used to study protein molecules in solution, as it offers insights into protein size, conformation, aggregation state, and crystallization potential [37, 38]. To explore the role of Mg^{2+} and Ca^{2+} in actin polymerization, light scattering experiments were performed on native APL and EDTA-treated APL. After adding actin polymerization buffer (APB)—a solution typically used to trigger polymerization—at 0.4 minutes, the light scattering intensity of native APL rose sharply and leveled off after approximately 0.1 minute (Figure 4). In contrast, EDTA-treated APL showed no change in scattering intensity upon APB addition, suggesting no polymerization occurred. These results demonstrate that magnesium and calcium ions in native APL are critical for enabling APB-induced protein polymerization.

This behavior aligns with mammalian actins, which also polymerize in the presence of APB [20, 39]. However, removing Mg^{2+} and Ca^{2+} from APL abolished its polymerization activity (Figure 4), confirming these ions are essential for the process. Similar observations have been reported for other actins: removing Mg^{2+} or Ca^{2+} irreversibly converts G-actin to an inactive form incapable of polymerization [12, 40]. Additional studies support the importance of Mg^{2+} in maintaining protein stability and biological function [41, 42]. We propose that EDTA-induced removal of Mg^{2+} and Ca^{2+} alters APL's structure, which primarily accounts for its loss of polymerization ability.

3.6 Predicted structure of APL

The trRosetta server is an online tool designed for quick and precise protein structure prediction, utilizing deep learning technology. In recent years, deep learning has become an essential tool for enhancing the accuracy of protein structure prediction, enabling the prediction of various proteins with complex structures [43]. As shown in Figure 5, the predicted structure of APL was composed mainly of α-helix, in accordance with the CD results. A great amount of loop structure play a key role in connecting the α-helix part. In Figure 5A, glutamic acid (Glu) was labeled in red, aspartic acid (Asp) was labeled in yellow, and histidine (His) was labeled in blue. The three kinds of amino acids have been reported to bind bivalent metal ions including Mg^{2+} and Ca^{2+}. Besides, these amino acids distribute at the exterior area of APL, or the regions between two helixes. Namely, they are commonly located at the non-rigid area, which makes it more flexible to bind metal ions. PyMol software was used to clarify the possible binding way of Mg^{2+} and Ca^{2+} to the residues. As shown in Figure 5B and C, the positively charged residues and His can cooperate to fix one metal ion. Besides, two adjacent Glu or Asp can also be bridged by a metal ion. There are a lot of such domains among the predicted structure of APL, which may explain why the actin in pork has such a high capacity to bind divalent metal ions.

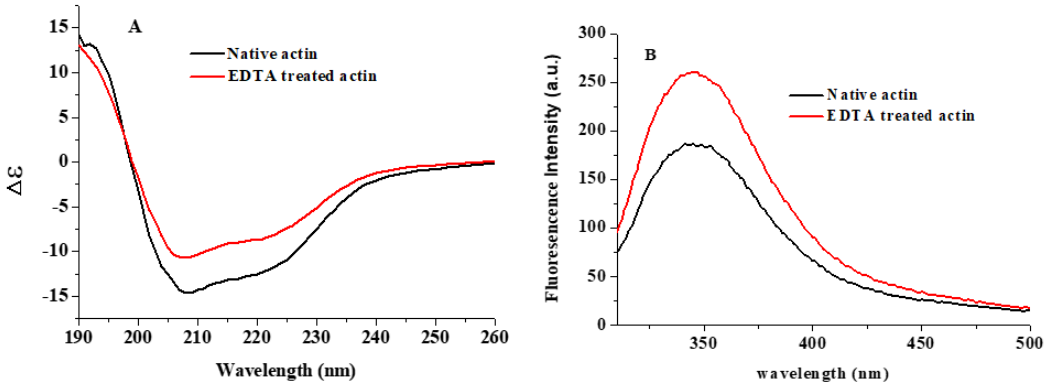

Figure 3 (A) CD spectra and (B) fluorescence emission spectra of EDTA-treated APL and native APL. Conditions: (A) EDTA-treated APL (1.5 μM) or native APL (1.5 μM) in 2 mM Tris-HCl (pH 8.0). (B) $\lambda_{Ex.}$ = 295 nm, slits for excitation and emission of 5 and 5 nm, respectively, 5.0 μM AGO, 50 mM Mops, pH 7.2, 25°C.

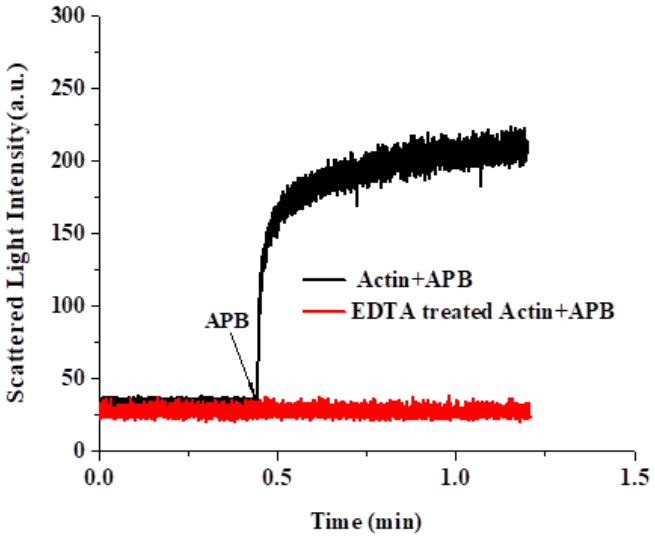

Figure 4 Scattered light intensity of APL (A) polymerization induced by action polymerization buffer (APB) as a function of time. The actin aggregation was initiated by mixing different types of actin and APB aerobically, and polymerization was followed by the intensity of scattered light at a 90° to the incident beam. Conditions: Both excitation wavelength and emission wavelength = 450 nm; [APL] = 5.0 μM in 2 mM Tris-HCl (pH 7.2), APB consists of 3 mM ATP, 2 mM Tris-HCl (pH 7.2), and 50 mM KCl, 25°C. All quoted concentrations are final concentration.

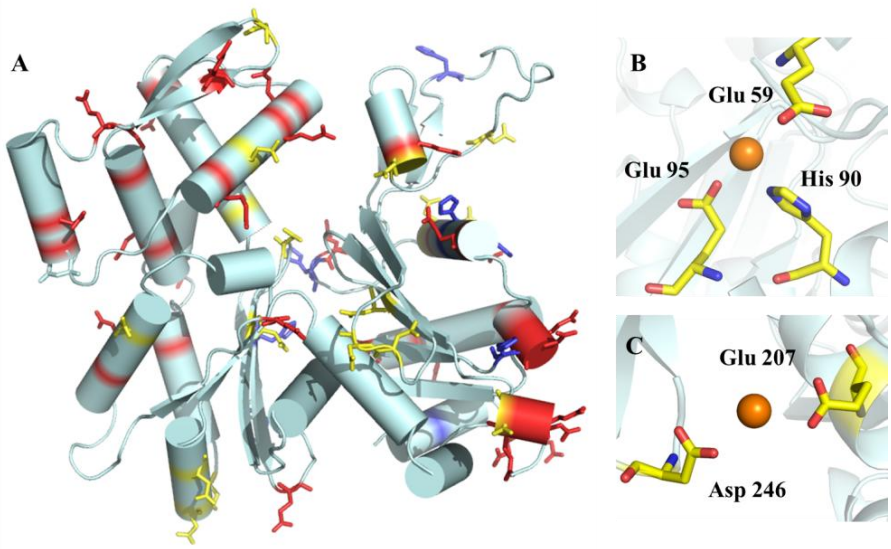

Figure 5 (A) predicted structure of APL. (B, C) possible binding ways between metal ions and APL.

The structural flexibility observed in APL's loop regions may allow for adaptive binding to different metal ions under varying physiological conditions. This adaptability could be particularly important in post-mortem muscle, where changes in pH and ion concentration influence protein-metal interactions [44]. Future studies could validate these predictions through mutagenesis of key residues followed by metal-binding assays. Additionally, molecular dynamics simulations could provide further insights into the dynamic interactions between APL and metal ions under different environmental conditions. Understanding these mechanisms could have implications for food science, particularly in optimizing meat processing techniques to enhance product quality.

4. Conclusion

A new non-muscular actin (APL) was isolated from porcine lung and purified to homogeneity for the first time, and its native state is a monomer with a molecular weight of approximately 42 kDa. APL contained all eight essential amino acids with abundant amounts. Different from all reported actins, APL exhibits a much higher activity of accumulating both magnesium and calcium. For example, each APL molecule contains approximately 33 atoms of magnesium and 18 atoms of calcium which have a marked effect on the structure and function of the protein. This new property of APL in accumulating minerals endows this protein with a great potential for exploration as magnesium and calcium supplement.

Abbreviations

APL	actin from porcine lung
SPI	soybean protein isolate
WP	whey protein
APB	actin polymerization buffer
G-actin	globular actin
F-actin	actin filaments
SDS	sodium dodecyl sulfate
EDTA	ethylenediaminetetraacetic acid
Tris	hydroxymethyl aminomethane
TEMED	tetramethylethylenediamine

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Author Contributions

Zeyu Liu was responsible for concept proposal, experimentation and writing original draft; Zhengyang Jia was responsible for data analysis and proofreading; Xuetong Wang was responsible for proofreading; Jiachen Zang was responsible for conceptualization, supervision, review and editing.

Competing Interests

No conflicts of interest exist.

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