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Quantitative Analysis of Triple-Helical Collagen Structure Using Affinity purification – Mass Spectrometry

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1. INTRODUCTION

Collagen is the most abundant fibrous structural protein in the human body, accounting for approximately 25% - 30% of the total protein content【1】. It is primarily secreted by fibroblasts【 2】and mainly present in tissues such as skin, bone, tendon, ligament, blood vessel, and cornea【3】. Additionally, collagen has also been identified in fish fins, fish skin, fish bone, fish scales, and fish swim bladder 【4,5】. Currently, 28 types of collagen are known to exist in the human body, which can be classified into different categories based on their structure and function, each exerting unique physiological roles 【6】.

The most characteristic feature of collagen molecules is their triple-helical structure, which consists of one triple-helical domain flanked by two non-helical regions at either end. The collagen triple helix (tertiary structure) represents a coiled configuration composed of three left-handed helical α-polypeptide chains (secondary structure) intertwined in a regular right-handed superhelical arrangement【7】, with structural stability primarily maintained through interchain hydrogen bonds and other interactive forces【8】. Additionally, several chaperone proteins, including prolyl 4-hydroxylase (P4-H), protein disulfide isomerase (PDI), an endoplasmic reticulum homologue of heat shock protein 70 (BiP/Grp78), various peptidyl-prolyl cis-trans isomerases (PPIase), and heat shock protein 47 (hsp47), also contribute to the stabilization of the triple-helical structure by preventing the α-chains from becoming entangled【9】. This unique triple-helix structure of the protein is capable of promoting cell growth and tissue repair【10】. Additionally, it can be absorbed by the body, exhibits non-toxicity, and demonstrates high biocompatibility, low antigenicity, and biodegradability 【11】【12】【13】【14】, among other physicochemical properties. As a result, it has been widely applied in the fields of biomedical materials, cosmetics, food, pharmaceuticals, and health【15】【16】【17】【18】

【19】. However, the triple-helical configuration, a pivotal marker of collagen's biological activity, is susceptible to detrimental effects from processing environments, which can lead to structural compromise and subsequent loss of biological functionality. Existing research has revealed that mutations or degradation of collagen are closely associated with various diseases, including osteogenesis imperfecta, Ehlers-Danlos syndrome, Alport syndrome, epidermolysis bullosa, and cancer【20】【21】. Therefore, the detection of these collagen variants is crucial for a better understanding of their roles in physiological and pathological processes, and it also offers potential avenues for disease diagnosis and treatment.

Existing detection methods mainly measure total collagen content without differentiating between triple-helical and non-triple-helical structures. Previous research has attempted to quantify triple-helical collagen using methods like trypsin digestion and hydroxyproline analysis, but these approaches fail to distinguish between different collagen types.

To address these limitations, this study introduces an innovative mass spectrometry-based method for the quantitative detection of collagen's triple-helical structure. The method involves extracting target collagen using specific antibodies, followed by enzymatic hydrolysis and mass spectrometry analysis to quantify specific peptide segments. This approach not only enables precise quantification of collagen's triple-helical structure but also allows for the differentiation of various collagen types, providing a more accurate and versatile tool for collagen research and application.

2. MATERIALS AND METHODS

2.1 Materials

Human skin fibroblasts (HFF-1 cells)

Trypsin (Promega, catalog number: V528A)

Lys-C

Various buffers (UA Buffer, Wash Buffer, Elution Buffer)

Antibodies (COL1A2 antibody, IgG antibody)

IP lysis buffer containing 20mM Tris (pH7.5), 150mM NaCl, 1% Triton X-100, and various inhibitors (sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, and leupeptin)

4% formaldehyde solution as a crosslinking agent

5M glycine solution for crosslink termination

Other common laboratory reagents and materialsMethods

2.2 Methods

2.2.1 Sample Preparation

Human skin fibroblasts (HFF-1 cells) were cultured. After culturing, the cells were treated with 4% formaldehyde for crosslinking to fix collagen's triple-helical structure. The cells were then lysed using IP lysis buffer under non-denaturing conditions. The lysate was centrifuged to obtain the supernatant containing the target collagen.

2.2.2 Antibody Binding and Extraction

The specific antibody COL1A2 was added to the supernatant to form an antigen-antibody complex with type I collagen. Protein A/G agarose beads were used to precipitate and separate the complex, obtaining a precipitate containing the target collagen.

2.2.3 Enzymatic Hydrolysis

The precipitate underwent three sequential enzymatic hydrolysis steps: first with trypsin, then with a mixture of trypsin and Lys-C, and finally with trypsin alone. These steps aimed to obtain specific peptide segments corresponding to the target collagen.

2.2.4 Mass Spectrometry Detection

The enzymatically hydrolyzed peptides were desalted and recovered. The desalted peptides were then analyzed using mass spectrometry. The mass spectrometry data were processed using pFind (V3.1.6) software to identify and quantify the specific peptides of collagen's α1 and α2 chains.

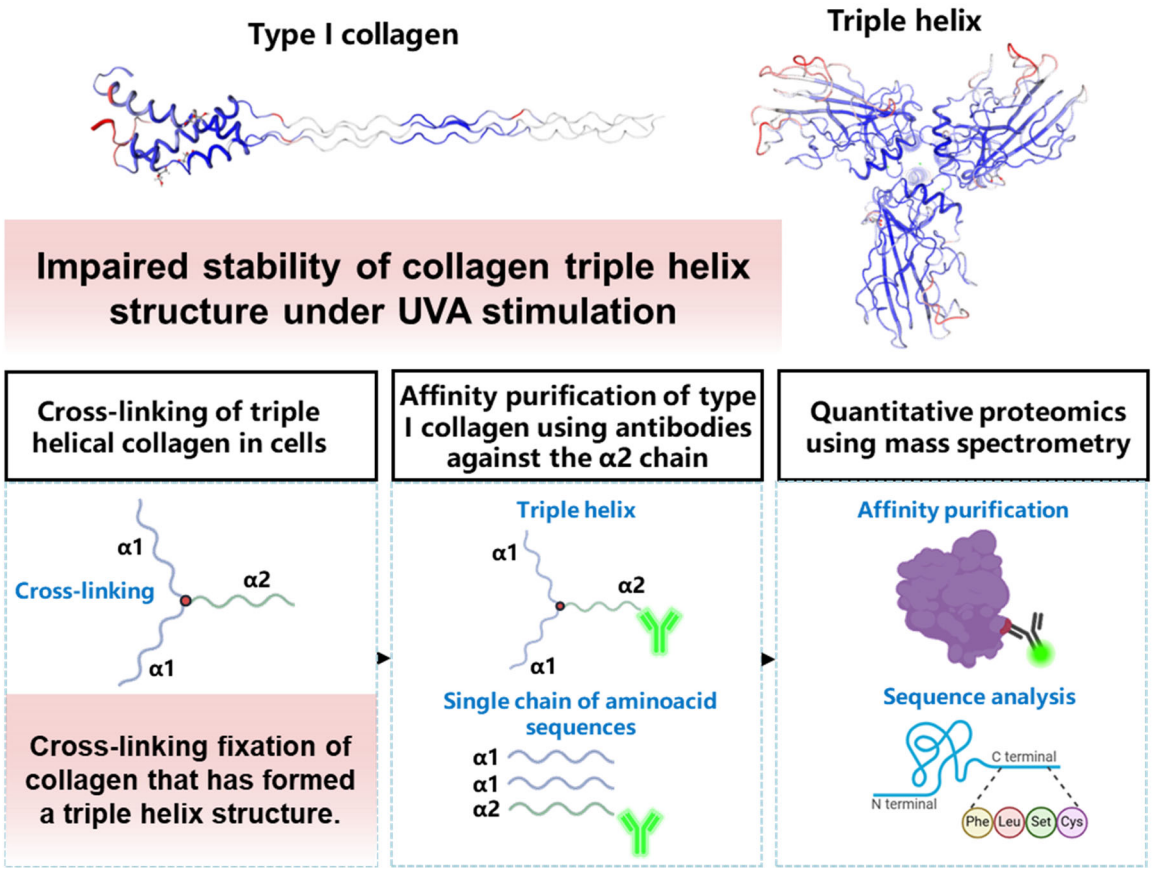
2.2.5 Data Analysis

Based on the identified and quantified specific peptides, the amount and proportion of collagen's triple-helical structure were calculated using specific formulas:

Triple-helical structure amount=(α1+α2)/2

Triple-helical structure proportion=α1+α2/[2×(α1+α2)]

Where $\alpha 1$ represents the specific peptide count of the α1 chain, and $\alpha 2$ represents the specific peptide count of the α2 chain.



3. RESULTS

The experimental results of this study provide compelling evidence for the significant impact of N-Acetylneuraminic acid (Sialic acid, SA) on the stability of collagen's triple-helical structure,

as well as the detrimental effects of UVA exposure. These findings were further supported by the detailed analysis of collagen triple-helical structures using the innovative mass spectrometry-based method described in this study.

3.1 Effect of SA Treatment on Collagen Stability

Compared to the control group, which exhibited a baseline triple-helix proportion of 60.54%, the SA-treated group showed a substantial increase in the triple-helix proportion to 81.3% (Figure 3). This significant enhancement suggests that SA treatment promotes the binding between collagen α1 and α2 chains, thereby stabilizing the triple-helical structure. The proteomics results (Figure 1) further revealed that SA treatment upregulates the expression of several key proteins involved in maintaining collagen stability, including P4HA1, P4HA2, P4HA3, and HSP47. These proteins play crucial roles in collagen biosynthesis and stabilization, indicating that SA may enhance collagen stability through multiple pathways.

3.2 Protective Effect of SA Against UVA-Induced Damage

Interestingly, when fibroblasts were pre-treated with SA before UVA exposure (UVA + SA-treated group), the triple-helix proportion was partially restored to 78% (Figure 3). This result suggests that SA may offer a protective effect against UV-induced damage, potentially through its ability to enhance collagen stability and mitigate the disruptive effects of UVA exposure.

3.3 Mass Spectrometry Analysis of Collagen Triple-Helical Structures

The mass spectrometry-based method used in this study allowed for precise quantification of collagen's triple-helical structure by analyzing specific peptides from the α1 and α2 chains. The results showed that SA treatment significantly increased the binding between the α1 and α2 chains of type I collagen, as evidenced by the higher number of triple-helical structures detected by affinity purification mass spectrometry (AP-MS) (Figure 2). This method also demonstrated its ability to differentiate and quantify various types of triple-helical collagen, providing a powerful tool for collagen research and application.

The experimental results demonstrated that compared to the control group with a triple-helix proportion of 60.54%, the N-Acetylneuraminic acid (Sialic acid, SA)-treated group exhibited a significant increase in the triple-helix proportion to 81.3%. In contrast, UVA exposure disrupted the collagen structure, resulting in a decrease in the triple-helix proportion to 38.8% in the UVA-irradiated group. However, in the UVA + SA-treated group, where fibroblasts were exposed to UVA after SA treatment, the triple-helix proportion was elevated to 78%.

FIGURE LEGENDS

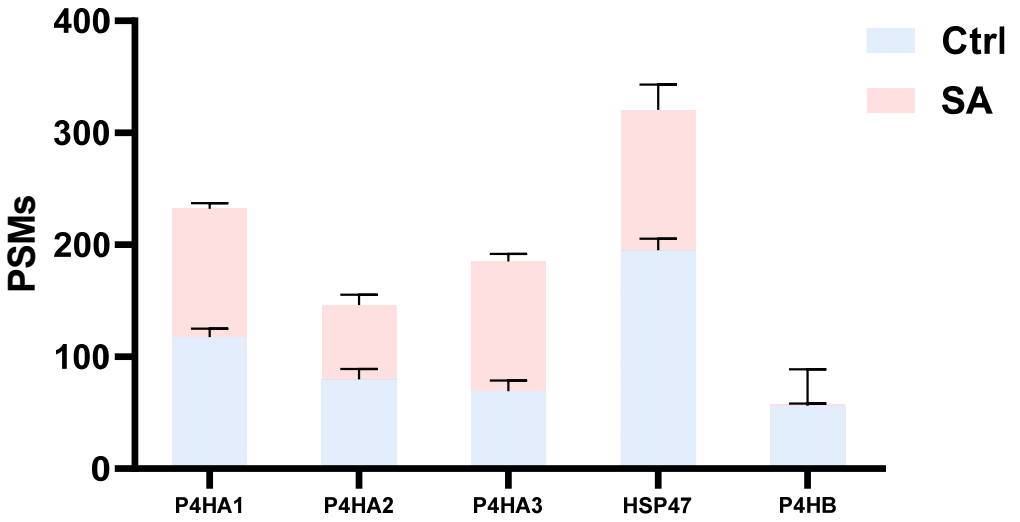


Figure 1. SA promotes the expression of multiple collagen structural stability-related factors. Proteomics results show that SA promotes the expression of P4HA1, P4HA2, P4HA3, HSP47 and other proteins, which play a key role in maintaining the stability of the collagen triple helix structure.

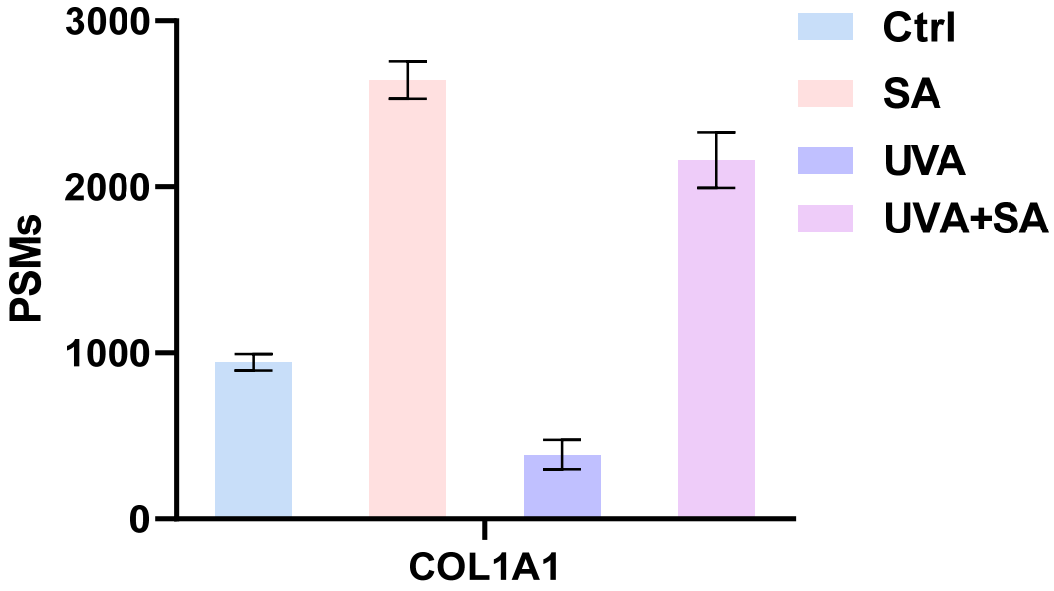


Figure 2. The number of triple helical structures of COL1A1 was detected by AP-MS. In collagens that form triple-helical structures, affinity purification of the α2 chain with an antibody purifies the α1 chain at the same time, so the number of peptides of the α1 chain detected by the final mass spectrometry determines the number of triple-helical structures.AP-MS results showed that SA significantly increased the binding between the α1 and α2 chains of type 1 collagen and improved the stability of the collagen triple helix structure.

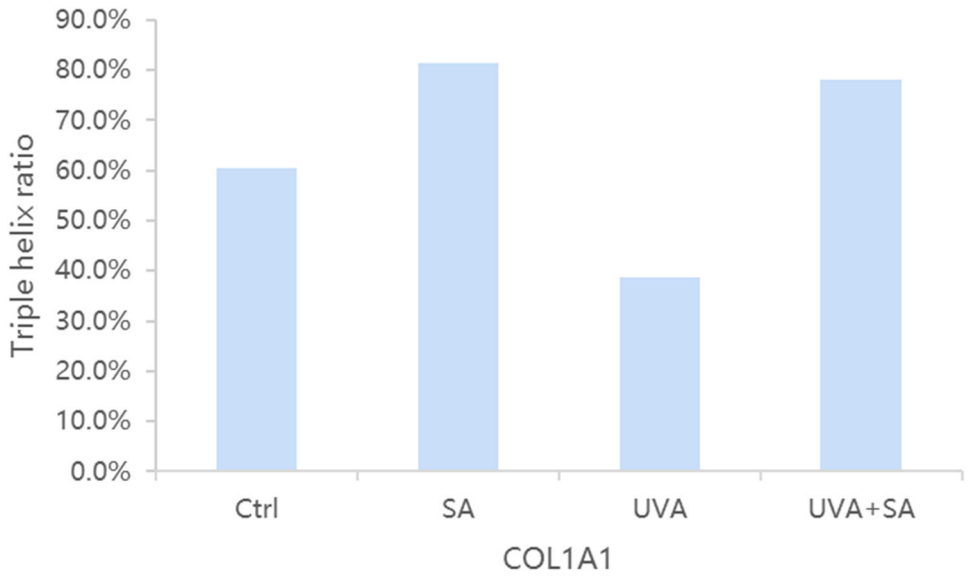


Figure 3. The proportion of the triple helix structure of COL1A1 was detected by AP-MS. The detection results by this method show that, compared with the blank control group, the proportion of the triple helix structure of collagen protein significantly increased after SA treatment, while it significantly decreased after UVA treatment. This is consistent with the phenomenon that sialic acid can promote the synthesis and secretion of collagen protein by fibroblasts, and UVA can disrupt the triple helix structure of collagen protein.

4. DISCUSSION

The findings of this study provide valuable insights into the stability of collagen's triple-helical structure and the potential role of N-Acetylneuraminic acid (Sialic acid, SA) in enhancing this stability. The results demonstrate that SA treatment significantly increases the proportion of collagen with a triple-helical structure, suggesting that SA may enhance the binding force between collagen α1 and α2 chains. This stabilizing effect could be attributed to SA's ability to form additional hydrogen bonds or other stabilizing interactions within the collagen molecule, thereby reinforcing the triple-helical conformation.

The observed decrease in the triple-helix proportion in the UVA-irradiated group is consistent with previous studies indicating that UVA exposure can lead to collagen denaturation and degradation. UV radiation is known to induce oxidative stress and generate reactive oxygen species (ROS), which can damage collagen fibers and disrupt the triple-helical structure. However, the partial recovery of the triple-helix structure in the UVA + SA-treated group suggests that SA may offer a protective effect against UV-induced damage. This finding has significant implications for the development of skin care products and therapies aimed at preventing or repairing UV-induced skin damage.

Furthermore, the innovative mass spectrometry-based method introduced in this study provides a powerful tool for quantitatively assessing collagen's triple-helical structure and evaluating the effects of various treatments on its stability. This method allows for precise

quantification of collagen's triple-helical structure and differentiation of various collagen types, offering a more accurate and versatile tool for collagen research and application. Future research could explore the detailed mechanisms underlying SA's stabilizing effects and investigate its potential in clinical and industrial settings, particularly in the development of collagen-based biomaterials and therapeutic agents.

5. CONCLUSION

In conclusion, this study demonstrates the potential of SA as a stabilizing agent for collagen's triple-helical structure. The mass spectrometry-based method developed in this study provides a robust and precise tool for quantitatively analyzing collagen's triple-helical structure and evaluating the effects of various treatments on its stability. The findings suggest that SA treatment can enhance the binding force between collagen α1 and α2 chains, thereby improving the stability of the triple helix structure. This effect is particularly evident in the context of UV-induced damage, where SA treatment partially mitigates the disruptive effects of UVA exposure. These results highlight the potential of SA as a protective agent against collagen degradation and offer new insights into strategies for enhancing collagen's stability and functionality in various applications. Future research should focus on elucidating the detailed mechanisms of SA's stabilizing effects and exploring its potential in clinical and industrial applications, particularly in the development of collagen-based biomaterials and therapeutic agents

REFERENCES

1. J. Myllyharju E.J. Massaro, J.M. Rogers (Eds.), *The Skeleton,* 1, Humana Press, Totowa, NJ (2004), pp. 77-87

2. Guillard J, Schwörer S. Metabolic control of collagen synthesis. *Matrix Biol*. 2024 Nov;133:43-56.

3. Zuber M, Zia F, Zia KM, Tabasum S, Salman M, Sultan N. Collagen based polyurethanes—A review of recent advances and perspective. *Int J Biol Macromol*. 2015 Sep;80:366-74.

4. Mahboob S. Isolation and characterization of collagen from fish waste material- skin, scales and fins of Catla catla and Cirrhinus mrigala. *J Food Sci Technol.* 2015 Jul;52(7):4296-305.

5. Liu D, Liang L, Regenstein JM, Zhou P. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (Hypophthalmichthys nobilis) .*Food Chemistry*. 2012 Aug;133(4):1441-1448.

6. Ricard-Blum S. The collagen family. *Cold Spring Harb Perspect Biol*. 2011 Jan 1;3(1):a004978.

7. Sorushanova A, Delgado LM, Wu Z, Shologu N, Kshirsagar A, Raghunath R, Mullen AM, Bayon Y, Pandit A, Raghunath M, Zeugolis DI. The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. *Adv Mater*. 2019 Jan;31(1):e1801651.

8. Shoulders MD, Raines RT. Collagen structure and stability. *Annu Rev Biochem*. 2009;78:929-58.

9. Koide, T., Nagata, K. Collagen Biosynthesis. In: Brinckmann, J., Notbohm, H., Müller, P.K. (eds) Collagen. *Top. Curr. Chem.*, vol 247,85.
10. Ayello E.A., Baranoski S., Kerstein M.D., Cuddigan J. Wound treatment options. In: Baranoski S., Ayello E.A., editors. Wound Care Essentials: Practice Principles. Lippincott Williams & Wilkins; Philadelphia, PA: 2003. p. 138.
11. Pati F, Datta P, Adhikari B, Dhara S, Ghosh K, Mohapatra PKD. 2012. Collagen scaffolds derived from fresh water fish origin and their biocompatibility. *J Biomed Mater Res Part A* :2012:100A:1068–1079.
12. Lynn AK, Yannas IV, Bonfield W. Antigenicity and immunogenicity of collagen. *J Biomed Mater Res B Appl Biomater.* 2004 Nov 15;71(2):343-54.
13. Yahyouche A, Zhidao X, Czernuszka JT, Clover AJ. Macrophage-mediated degradation of crosslinked collagen scaffolds. *Acta Biomater.* 2011 Jan;7(1):278-86.
14. Choi FD, Sung CT, Juhasz ML, Mesinkovsk NA. Oral Collagen Supplementation: A Systematic Review of Dermatological Applications. *J Drugs Dermatol.* 2019 Jan 1;18(1):9-16.
15. Ramshaw JA. Biomedical applications of collagens. *J Biomed Mater Res B Appl Biomater.* 2016 May;104(4):665-75.
16. Ramasamy P, Shanmugam A. Characterization and wound healing property of collagen-chitosan film from Sepia kobiensis (Hoyle, 1885). *Int J Biol Macromol.* 2015 Mar;74:93-102.
17. Inacio PAQ, Chaluppe FA, Aguiar GF, Coelho CF, Vieira RP. Effects of Hydrolyzed Collagen as a Dietary Supplement on Fibroblast Activation: A Systematic Review. *Nutrients.* 2024 May 21;16(11):1543.
18. Angre T, Kumar A, Singh AK, Thareja S, Kumar P. Role of Collagen Regulators in Cancer Treatment: A Comprehensive Review. *Anticancer Agents Med Chem.* 2022;22(17):2956-2984.
19. Avila Rodríguez MI, Rodríguez Barroso LG, Sánchez ML. Collagen: A review on its sources and potential cosmetic applications. *J Cosmet Dermatol*. 2018 Feb;17(1):20-26.
20. Myllyharju J, Kivirikko KI. Collagens and collagen-related diseases. *Ann Med.* 2001 Feb;33(1):7-21.
21. Necula L, Matei L, Dragu D, Pitica I, Neagu A, Bleotu C, Diaconu CC, Chivu-Economescu M. Collagen Family as Promising Biomarkers and Therapeutic Targets in Cancer. *Int J Mol Sci.* 2022 Oct 17;23(20):12415.