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Heterologous expression, fermentation strategies and molecular modification of collagen for versatile applications

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ABSTRACT

Collagen is a kind of high macromolecular protein with unique tissue distribution and distinctive functions in the body. At present, most collagen products are extracted from the tissues and organs of mammals or marine fish. However, this method exhibits several disadvantages, including low efficiency and serious waste generation, which makes it difficult to meet the current market demand. With the rapid development of synthetic biology and the deepening of high-density fermentation technology, the collagen preparation by biosynthesis strategy emerges as the times require. Co-expression with the proline hydroxylase gene can solve the problem of non-hydroxylated collagen, but the yield may be affected. Therefore, improving the expression through molecular modification and dynamic regulation of synthesis is an entry point for future research. Due to the defects in certain properties of the natural collagen, modification of properties would be benefit for meeting the requirements of practical application. In this paper, in-depth investigations on recombinant expression, fermentation, and modification studies of collagen are conducted. Also, it summarizes the research progress of collagen in food, medicine, and beauty industry in recent years. Furthermore, the future development trend and application prospect of collagen are discussed, which would provide guidance for its preparation and application.

KEYWORDS

Collagen, collagen modification, efficient expression, fermentation, gene co-expression

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Introduction

Collagen is the most abundant and widely distributed functional structural protein in invertebrates and vertebrates, accounting for about 30% of the total protein (Di Lullo et al. 2002). Collagen has a strong stretching ability, mainly in the skin, ligament, cartilage, blood vessels, teeth, bone, and other tissues, so these connective tissues have a strong mechanical tensile strength under physiological state. Collagen is widely distributed in the extracellular matrix and plays an active biological role in organ support, body protection, and tissue damage repair. It is an extremely important biological macromolecular protein in our body (Romero-Ortuno, Kenny, and McManus 2020). Collagen is a natural protein in the body. It has good biological and physical properties for protein molecules on the skin surface, such as high affinity, good adhesion, weak antigenicity, great biocompatibility, and biodegradability. It is widely used in food, medicine, and beauty fields, which has attracted increasing interests. Due to the broad application prospects and significant commercial value of collagen production, its industry market size is expected to exceed $6.6 billion by 2025 (Rodriguez, Barroso, and Sanchez 2018).

Predecessors' review articles mostly focused on structural research, traditional extraction as well as related application, while this is the first time to systematically review the efficient preparation of collagen from the perspective of biosynthesis. This article introduces the various levels of structure using the synthesis process of collagen in vivo as a narrative clue, and then, through comparison with traditional extraction methods, it emphasizes the strategy and prospects of biosynthesis combined with high-density fermentation technology to prepare collagen, followed by a

description of the modification and applications further reveals the hot market demand and development prospects of collagen.

Classification of collagen

Collagen is a special triple helix structure formed by the winding of three-stranded α polypeptide chains. Different collagen types are formed due to various combinations of the three polypeptide chains. To date, 29 types of collagens have been reported, of which type I, II, and III account for the largest proportion, all of which account for more than 90% of the total collagen in vivo (Sorushanova et al. 2019). According to the α polypeptide chains of collagen, it could be divided into homotrimers with three identical α chains (e.g., type II, type III collagen) and heterotrimers with two or three different α chains (e.g., type I, type IV collagen) (Figure 1) (Seo et al. 2017). On the other hand, collagen can be further divided into fibroblast collagen and non-fibroblast collagen according to their functions. Fibroblast collagen includes collagen types I, II, III, XI, XXIV, and XXVII, the rest are non-fibroblast. Non-fibroblast collagen includes reticulated collagen, fiber-related collagen, anchored collagen, and transmembrane collagen (Mienaltowski and Birk 2014).

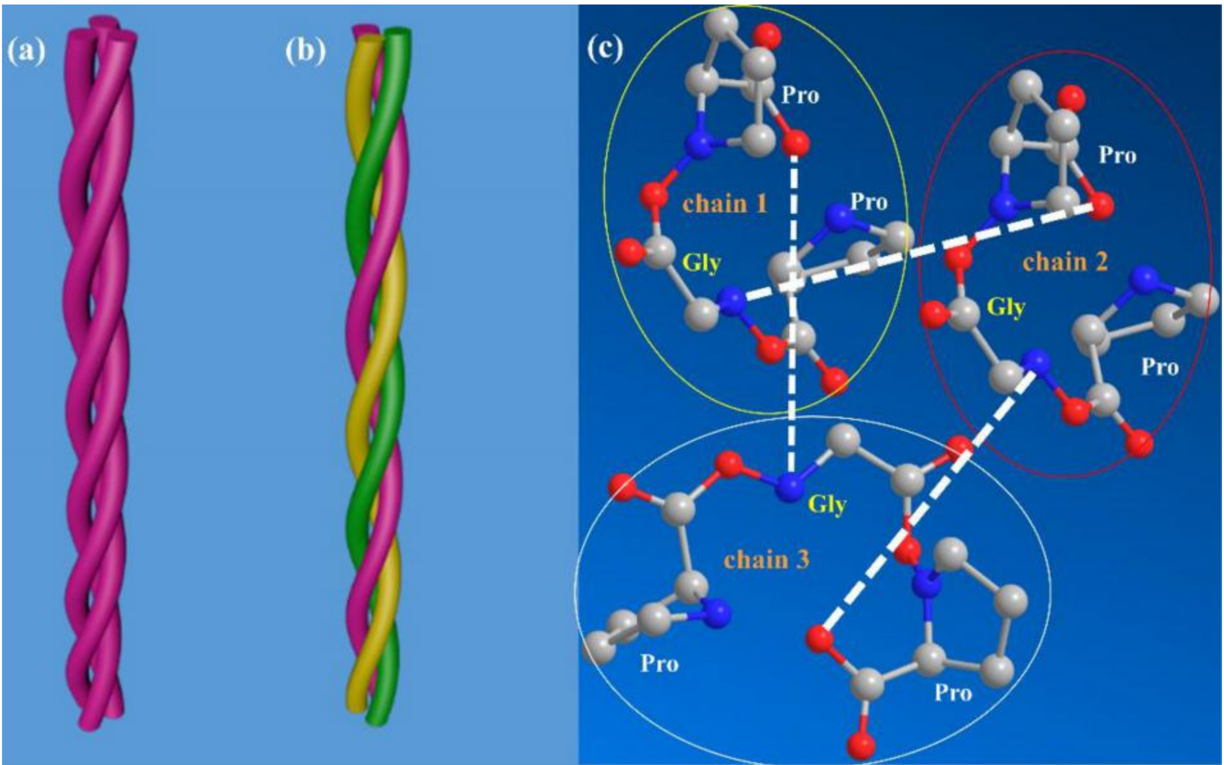


Figure 1 . Structure and types of collagens. (a) Homotrimers. (b) Heterotrimers. (c) The N-H of glycine forms hydrogen bond with the C=O of adjacent Pro residues.

The structure of collagen

The spatial structure of proteins is the basis of protein functions and characteristics. Collagen has been proven to be a natural polymer with a multi-layer structure (Liu et al. 2019).

Synthesis, assembly and maturation of collagen

Collagen is mainly synthesized and secreted by fibroblasts. Its synthesis, assembly and maturation could be regulated and modified by a series of intracellular and extracellular elements. The gene encoding the alpha chain of procollagen is translated into a propeptide chain on the ribosome after transcription, and then posttranslational modified and self-assembled to form procollagen with the formation of disulfide bonds between the chains. Procollagen is cleaved by extracellular procollagen peptidase at specific N

and C terminal sites to produce three fragments: N-terminal propeptide, C-terminal propeptide, and protocollagen. Protocollagen is a three-strand helical structure with a relative molecular weight of around 285000 Da, which is composed of three α peptide chains containing 1000 amino acid residues each, and finally, protocollagen could be covalently crosslinked and polymerized into mature collagen fibers (Liu et al. 2019; Seo et al. 2017).

In particular, much attention has been focused on this process by which collagen self-assembles to form superhelical structures. The C-terminal propeptide domain of collagen is rich in cysteine, which is responsible for initiating the folding, strand selection, and the establishment of a suitable triple helix register of the collagen (Prockop et al. 1979). As early as 1997, the chain discontinuous recognition sequence, which consists of 23 amino acid residues was reported, contributes to the self-assembly of collagen (Lees, Tasab, and Bulleid 1997). Recently, it was pointed out that the α1 chain of human collagen I can spontaneously assemble into homotrimers, and its C-terminal propeptide contains eight cysteines (C1-C8), whereas the α2 chain can only interact with other α chains to form heterotrimers, and its C-terminal propeptide has only seven cysteines. Where C2 is involved with C3 in the formation of interstrand disulfide bonds, while the remaining cysteines form intrastrand disulfide bonds (Sharma et al. 2017), thus, C2 and C3 in the C-terminal propeptide may play critical roles on collagen self-assembly. The work of Dichiara and coworkers reassesses the rationality of this hypothesis. They demonstrated that the formation of interstrand disulfide bonds by cysteine in the C-terminal propeptide of human type I collagen is a key link in regulating collagen selfassembly, and the interstrand disulfide bonds provide a thermodynamic sink to stabilize the non-covalent trimer, ultimately forming a three-strand superhelix (DiChiara et al. 2018). The specific mechanism of self-assembly is

complex, which has not been fully resolved, and further research and exploration are needed.

Multi-hierarchy structure of collagen

The basic structure of collagen is protocollagen molecule, which is composed of three-stranded α polypeptide chains. The chain in the protocollagen contains the unique amino acid sequence structure of collagen. Scientists found a large number of $(Gly\text{-}x\text{-}y)_n$ repetitive sequences in the α polypeptide chain through primary structure analysis. The Gly content is up to 30%, and x often is proline (Pro), while y is mostly hydroxyproline (Hyp) or hydroxylysine (Hyl) in the sequence. This unique structure is necessary for the formation of the advanced structure of collagen. Since the Pro in the Gly-Pro-Hyp triplet has a metastable structure, the conformations of the tetrahydropyrrole ring can be both endo and exo, and the internal-external alternation of the ring indicates the compression and expansion of the collagen triple helix. The metastable structure of Pro gives structural flexibility of collagen, that is, the flexibility at the molecular level confers the flexibility at the structural fiber level (Goldberga, Li, and Duer 2018).

Hyp also plays an extremely important role in the synthesis of collagen, which accounts for about 10% of the amino acid residues of collagen, is the hallmark residue of collagen molecules; therefore, Anna Sorushanova et al. (2019) called Hyp a molecular fingerprint of collagen. Hyp residues are closely related to the thermal stability of the triple helical structure of collagen (Gu et al. 2019). As early as 1998, researchers have demonstrated that Hyp stabilizes the triple helical structure through the formation of intramolecular hydrogen bonds by synthesizing halogen-substituted peptide $(ProFlpGly)_{10}$, which endows the thermal stability of collagen (Holmgren et al. 1998). In addition, since the side chains of Hyp and Pro molecular structures are circular, the bonds between the α carbon and amide nitrogen

cannot be arbitrarily rotated, which is conducive to the formation of left-handed helical peptide chains in collagen (Brodsky and Ramshaw 2017).

The secondary structure of protein refers to the regular local spatial conformation produced by the main chain folding of the peptide chain. The secondary structure of collagen is the left-handed α helix formed by the electrostatic interaction between Pro and Hyp, and the hydrogen bond between amino acid residues maintains the stability of the α helix structure.

The further interwinding of the three α chains forms a specific triple helix structure, which is the basic morphology of procollagen. C-procollagenase hydrolyzes down the three additional peptide segments with disulfide bonds at the carboxyl terminus of procollagen simultaneously, turning the procollagen into the N-procollagen with only an amino terminal additional peptide, and then, the N-procollagenase specifically excises the amino terminal additional peptides segments of N-procollagen to form protocollagen, but the specific cleavage mechanism is unclear. This triple helical structure is a right-handed superhelical cable whose values of dihedral angle φ and ψ are about −60° and +140°, respectively. The collagen helix is much more extended than the α helix, with a pitch of 8.6 nm and contains about 30 residues. Every two residues in the α chain of the collagen triple helix, that is, the third amino acid residue, are located around the narrow and crowded central axis. Glycine becomes the only suitable residue because of the smallest volume, and the residues at both ends of glycine are located on the outside of the helix (Liu et al. 2019). In the collagen helix, the N-H of glycine forms hydrogen bond with the C=O of adjacent Pro residues, and the hydroxyl of Hyp also participates in the formation of inter-chain hydrogen bond, which leads to the favorable stability of the triple helix (Figure 1).

Collagen, also known as collagen fibers, exists in the body as a supramolecular aggregated structure. Collagen triple helix would spontaneously gather and assemble into a network structure according to a

quarterly staggered manner in the body, thus forming collagen fibers with a rather crystallographic structure (Liu et al. 2019). In other words, under the action of van der Waals force and hydrogen bond, collagen molecules will crisscross regularly and arrange in parallel from head to tail, forming a high-level supramolecular structure, and finally showing the morphological characteristics of collagen fibers which are extremely similar to the classical "rope" structure (Bozec, van der Heijden, and Horton 2007). The synthesis process of collagen is shown in Figure 2.

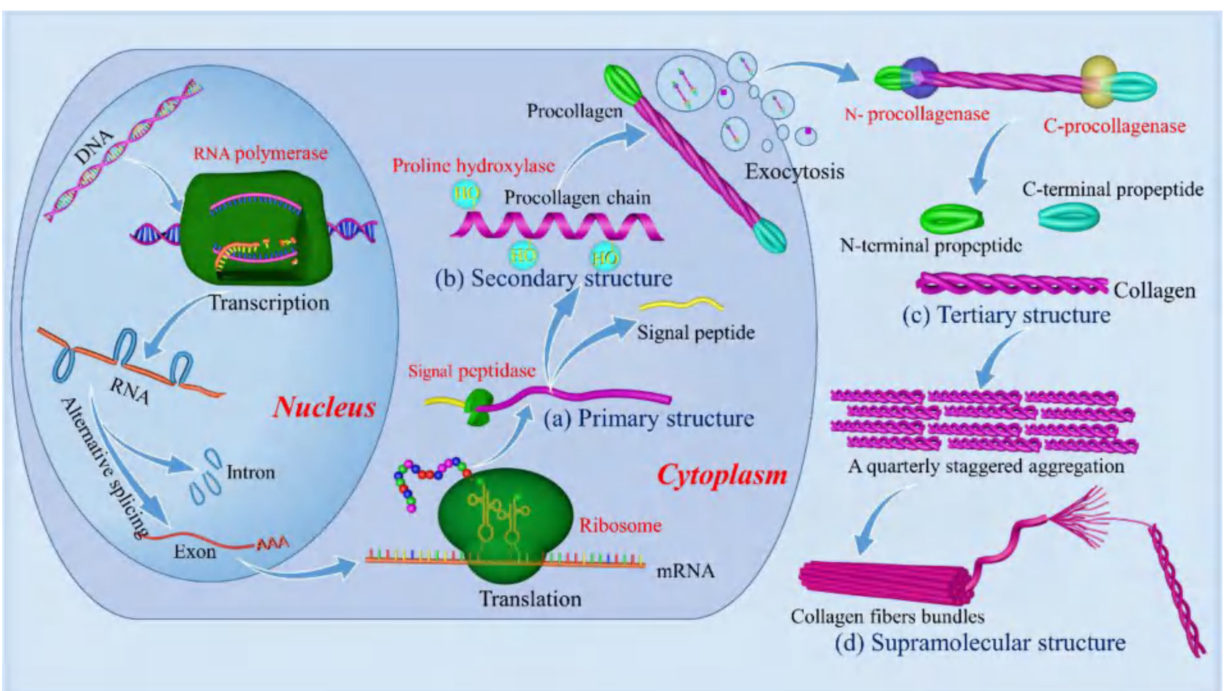


Figure 2. The expression and synthesis process of collagen. (a) Amino acid sequence of procollagen (primary structure). (b) The electrostatic repulsion between Pro and Hyp forms left-handed *α* helix (secondary structure). (c) Three-strand *α* helix chain assembled into right-handed super-helix structure (tertiary structure). (d) Collagen fiber bundles (supramolecular structure).

Preparation methods of collagen

The traditional preparation method of collagen is mainly obtained by extracting from animal’s connective tissue by acid hydrolysis, alkali hydrolysis, or enzymatic method.

Acid hydrolysis

The method of acid hydrolysis is to use low concentration organic acid to destroy the salt bond and Schiff base in the molecular structure, causing the expansion and dissolution of collagen fiber. Acid extraction has been reported to not destroy the structure of natural collagen and the extracted purity is very high (Caputo et al. 2012). Acetic acid is the most commonly used organic acid extractant due to its high extraction efficiency (Hong et al. 2019). Recently, researchers have employed acetic acid hydrolysis to extract collagen from the skin of Nile tilapia. The author investigated the optimal extraction conditions from the three factors of acetic acid concentration, extraction temperature and extraction time. Eventually, the yield of 19% was obtained under the conditions of 0.35 mol L^{-1} acetic acid concentration and 20 °C extraction temperature for 65 h (Menezes et al. 2020), which is similar to a previous report that 19.3% yield was obtained from the skin of sole fish with acetic acid/NaCl solution (Arumugam et al. 2018).

Alkali hydrolysis

Alkaline preparation is to treat collagen with a certain concentration of alkali to break the peptide chain and dissolve collagen. The commonly used treating agents in alkali treatment are lime, NaOH, Na_2CO_3, and other alkaline reagents (Hong et al. 2019). Tilapia skin was treated with 0.12 mol L^{-1} NaOH solution for 24 h at room temperature, and the yield was 38.8% (Bi et al. 2019). The alkali method will destroy the hydroxyl, sulfhydryl groups in collagen and even lead to racemization and protein denaturation. There are few reports about the collagen preparation by alkali hydrolysis alone and most of them are prepared by combining with the acid-base method. The grass carp skin was pretreated with 0.05-0.1 mol L^{-1} sodium hydroxide and then pretreated with 0.5-1.0 mol L^{-1} acetic acid at 4-20 °C,

the acid soluble collagen with complete structure and high yield can be obtained (Liu et al. 2015).

Enzymatic method

The extraction of collagen by acid and alkali method will reduce the quality of the target product and generate a large number of byproducts, resulting in wastage of raw materials. Compared with the two methods above, the enzymatic method not only reduces the processing time, but also greatly improves the extraction yield and quality, as well as reduces the waste. However, the enzymatic method has strict requirements on pH and temperature (Ahmad et al. 2017). Treated chicken feet with papain at 30 °C for 28 h, the yield reached 32.16% with high quality (Araujo et al. 2018). Xu et al. (2021) extracted type II collagen from chicken sternal cartilage by two methods-cryotherapy with pepsin and without pepsin treatment, to obtain pepsin soluble undenatured type II collage (SC II) and natural insoluble undenatured type II collagen (IC II). The solubility and purity of SC II in the total proteins extracted were higher than IC II, which the proportion of soluble protein and the content of type II collagen in SC II were 82.55% and 75.62%, respectively, while in IC II was only 2.25% and 60.73%. What is more interesting is that the soluble undeformed type II collagen in SC II is 1.2-12.4 times higher than IC II after digested by the gastrointestinal tract. In order to enhance the extraction efficiency, the combination of enzymes might be a promising candidate for extraction experiments over the single enzyme. In literature, it has been proven that the "pepsin-trypsin" double enzyme system for treating the viscera of silver carp could produce lower molecular weight collagen peptides compared with pepsin alone (Abdollahi et al. 2018).

Collagen contains a large number of covalent cross-linked structures, which makes low solubility. Since the appropriate addition of some proteases can effectively destroy the cross-linked structure to increase

solubility and yield, enzymatic pretreatment can be used before acid or alkali extraction. Based on the complex enzyme methods proposed by predecessors (Nagai and Suzuki 2000; Rousseau and Gagnieu 2002; Saito et al. 2002), Li et al. (2020) obtained 72.2%, 67.3%, and 64.7% of yields from sea cucumber, Tilapia skin, and pig skin by pepsin, respectively.

Novel approaches

In recent years, using ultrasonic-assisted acid treatment (Zou et al. 2017) has significantly improved the extraction efficiency, and the application of the new technology brings new breakthroughs for the extraction of collagen. Wang, et al. (2020) employed supercritical carbon dioxide ($SCCO_2$) extraction technique to decellularize pretreated porcine skin to prepare collagen matrix scaffolds for wound healing and skin burn treatment. Pigskin contains a large amount of collagen, and the $SCCO_2$ technique is to decellularize porcine skin under the critical temperature and pressure, and it can remove lipids and other hydrocarbons, while retaining polar molecules such as proteins, and eventually obtain collagen matrix with noncytotoxic as well as appreciable biocompatibility. This supercritical extraction technique is intrinsically different from previous extraction methods, which opens up new ideas for the extraction of collagen and other active substances in organisms.

Conventionally, most of the collagen in the market was extracted from connective tissues of the animal; however, the products from extraction procedures are generally water-insoluble proteins with complex ingredients and partial loss of biological activity. In clinical practice, there might be immune rejection, and the risk of animal-derived pathogens (such as prions, foot-and-mouth disease virus, HIV, rabies virus). Also, the heavy metal poisoning should be considered, thus the quality control of products is not easy (Li et al. 2020; Lynn, Yannas, and Bonfield 2004). On the other hand, the influence of religious belief for the specific application of the extracted

collagen from certain mammals must also be taken into account. For example, Muslims refuse to use pork collagen products (Easterbrook and Maddern 2008). This has led scientists to turn their attention to marine organisms in order to extract collagen from marine fish (Subhan et al. 2021; Wan et al. 2021). Although marine biological materials can reduce the above risks to a certain extent, there are still challenges such as the carrying of certain pathogens and excessive heavy metals, which limits the extensive application of collagen.

Efficient expression of collagen

Since traditional extraction methods could not meet various market demands, heterologous expression of recombinant collagen using molecular biology and genetic engineering technology has become a promising choice for its in-depth application. Microbial production of collagen not only overcome the deficiencies in the traditional preparation process, but also simplify the production process and reduce the production costs. In 1991, scientists successfully expressed human type II procollagen in Mouse 3T3 cells (Ala-Kokko et al. 1991). As the development of genetic engineering and high-density fermentation technology, substantial progress has been made in the efficient expression of collagen, especially human collagen.

The heterologous expression strategy is not only able to activate the expression of certain silent genes but also to increase the yield of low-producing compounds to efficiently produce valuable biomolecules in a more suitable heterologous host (Huo et al. 2019). Appropriate molecular modification on the basis of heterologous expression to enhance production potential at the strain level, and coupled with high-density fermentation to further achieve high yields of the target products, which has emerged as an extremely efficient mean of collagen production processes. Table 1 lists the investigations on heterologous expression of recombinant collagen in recent years.

Table 1. Expression of recombinant collagen in various expression systems. (Table view)

Expression hosts	Expression vectors	Types	Hydroxylation	Expression level	Molecular weight(kDa)	Refere
Escherichia coli (E. coli)	pJY-1/pJY-2	Human-like collagen	Non-hydroxylation	$0.1\sim0.2$ g L^{-1}	73.3	(Yin et 2003
	pET30a(+)	Human-like collagen	Non-hydroxylation	$50\sim70$ mg L^{-1}	32	(Yao Yanagis and Asa 2004
	pET28a/pET16b	Human collagen (III)	Hydroxylation	90 mg L^{-1}	38	(Rutschi et al. 2
	pACDuet-1	Human-like collagen	Hydroxylation	0.26 g L^{-1}	35	(Tang e 2016
	pKK223-3	Human collagen (III)	Hydroxylation	/	110	(Shi et 2017
Pichia pastoris (P. pastoris)	/	Human collagen (I, II, III)	Hydroxylation	$0.7\sim1.5$ g L^{-1}	/	(Olsen 2003
	pPIC9K	Human collagen (III-α1)	Non-hydroxylation	1.27 g L^{-1}	120	(Wang 2014
	pPIC9K	Human collagen (α1)	Hydroxylation	300 mg L^{-1}	140	(Mi et 2018
	pPIC9K	Human collagen (III-α1)	Non-hydroxylation	4.68 g L^{-1}	130	(Li et 2015
Tomato	EHA105	Human collagen (I)	Hydroxylation	20 g L^{-1}	86	(Stein et 2009)
Corn	T-DNA	Human collagen (I)	Hydroxylation	12 mg kg^{-1}	/	(Xu et a 2011)
Silkworm	pMSG1	Human collagen (I)	Non-hydroxylation	80 g kg^{-1}	120	(Adachi 2010)

Recombinant expression in E. coli system for collagen production

At present, the most widely used protein expression system is the E. coli expression system. Due to its clear expression mechanism, explicit genetic background, low fermentation cost, high expression efficiency, simple culture, and short cycle, E. coli is one of the most successfully applied expression systems for the large-scale production of foreign proteins. Human collagen expressed by recombinant E. coli mainly consists of hydroxylated and non-hydroxylated collagen.

Non-hydroxylated collagen

Prolyl hydroxylase is a key enzyme in the synthesis of collagen, catalyzing the formation of 4-Hyp, and then enables collagen to form stable triple helix (Figure 3). The E. coli expression system lacks prolyl hydroxylase and the expressed foreign proteins are generally non-hydroxylated collagen. The researchers cloned and expressed the human-like collagen gene in E. coli, and the expression quantity eventually reached $50 \sim 70$ mg L^{-1} (Yao, Yanagisawa, and Asakura 2004). In some cases, T7 and T7 lac promoters were employed to induce expression by IPTG in order to improve the expression level, and finally obtained non-hydroxylated collagen of $0.1 \sim 0.2$ g L^{-1} (Yin et al. 2003).

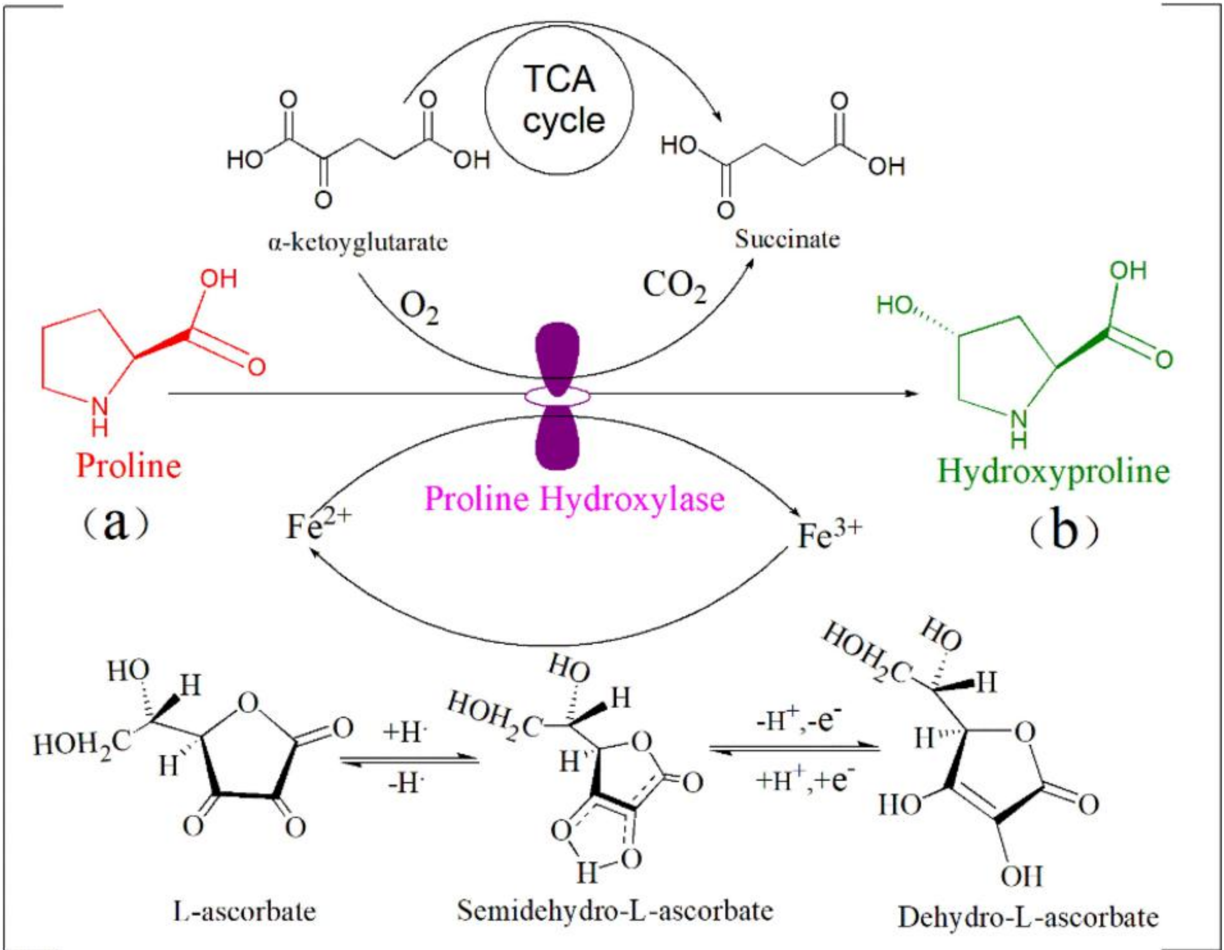


Figure 3. Proline hydroxylase catalyzes Pro production into Hyp. (a) Pro. (b) Hyp. In the catalysis process, *α*-ketoglutarate is needed as auxiliary substrate, and O_2, Fe^{2+} and ascorbic acid are needed as auxiliary factors. Hypoxic conditions would affect the activity of hydroxylase and ascorbic acid plays a reductive role in maintaining the Fe^{2+} which is required by hydroxylase.

Co-expression strategy for direct synthesis of hydroxylated collagen

Collagen without hydroxylation is difficult to form triple helix because of the lack of residues that can be covalently cross-linked, thus it belongs to denatured collagen theoretically. This non-hydroxylated collagen cannot meet the application requirements in many aspects, especially in the medical field. A gene co-expression strategy has been proposed in order to express hydroxylated collagen in E. coli system in literature. The expression vectors containing collagen gene, lysyl hydroxylase gene, and

prolyl hydroxylase gene were transformed into E. coli, and finally resulting in the successful expression of hydroxylated collagen (Rutschmann et al. 2014).

Afterwards, Tang et al. (2016) co-expressed the proline hydroxylase gene, d-arabinose 1, 4-lactone oxidase gene, and human-like collagen gene in the E. coli system, and 0.26 g L^{-1} hydroxylated collagen was obtained by 0.1 mM IPTG induction. The co-expression strategy effectively solves the problem of hydroxylation. In another case, co-expression of P4H gene and type I collagen gene was attempted to promote the secretion of mature collagen. The results showed that mouse embryonic fibroblast (MEF) cells treated with ascorbic acid for 3-6 hours did not up-regulate the mRNA transcription level of type I collagen α2 chain (COLI α2); however, it enhanced glycosylation of α1 subunit of P4H at N_{259}, which could induce hydroxylation of P4H to Pro. This study further confirmed that the up-regulation of N_{259} glycosylation was post-translationally modified by the magnesium transporter ($MAGT_1$) and STT_3B complex ($MAGT_1/STT_3B$), which could promote procollagen to enter the Golgi apparatus for processing and modification, and finally promote the secretion of mature COLI α2 (Shi et al. 2019b). This phenomenon also verifies the previous conclusion that ascorbic acid can stimulate the biosynthesis of procollagen through the association between the endoplasmic reticulum and ribosomes (Ueno et al. 2010).

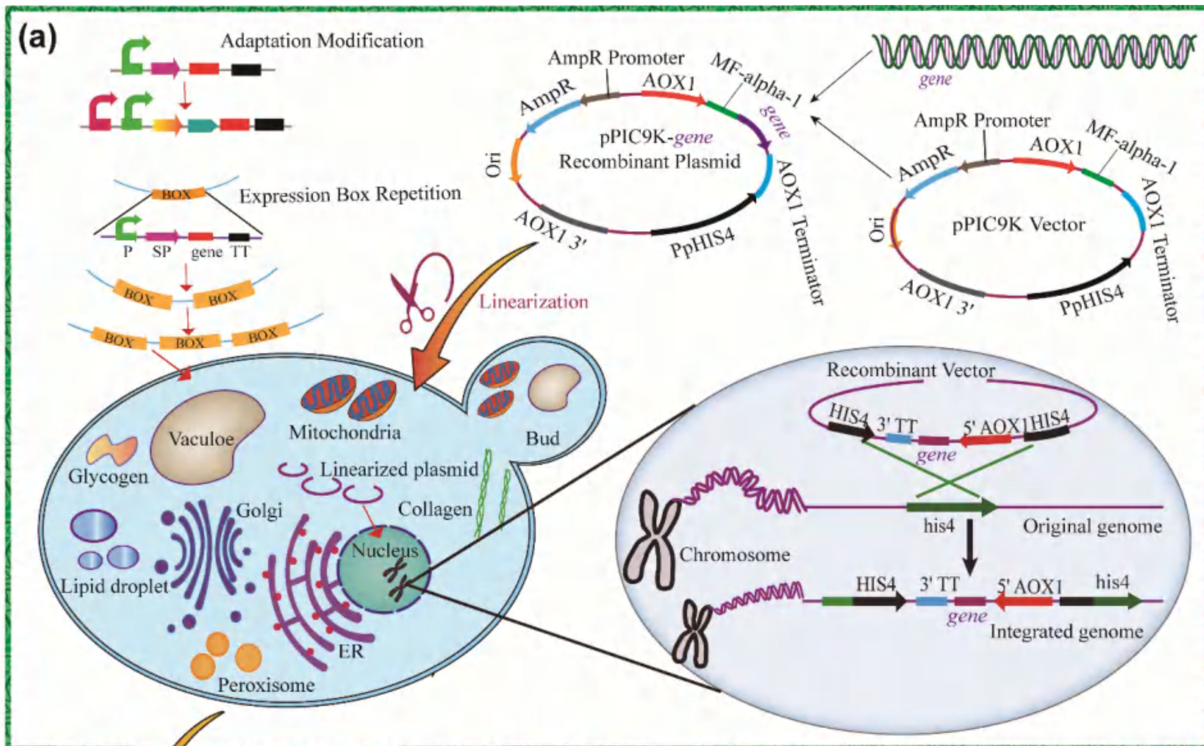
In some cases, co-expression strategies might may reduce the expression level of target proteins. Previously, Stein et al. (2009) compared the expression of the type I collagen gene alone with the co-expression of collagen gene and P4H gene in the endosperm cells of corn. It was found that the hydroxylation rate of the co-expression group was 18.11% but the expression level only reached 4 mg kg^{-1}. Whereas the yield of the independent expression group reached 12 mg kg^{-1}, which was 3 times higher than that of the co-expression group. Although hydroxylated human-

like collagen can be directly obtained through co-expression strategy, the co-expression may affect the expression level and final yield of collagen. In order to improve the yield, dynamic regulation of its synthetic pathways and rational modification of the two genes might be required to synthesize the hydroxylated collagen efficiently and directly.

Recombinant expression in P. pastoris system for collagen production

As a prokaryote, the post-translational modification system of E. coli is not perfect; also, it is easy to form inactive inclusion bodies in cells when foreign proteins are expressed. Besides, clinically, the culture process of E. coli may produce endotoxin.

The eukaryotic expression system, such as yeast, has a complete mechanism of post-translational modification, including glycosylation, hydroxylation, etc. Another obvious advantage of yeast expression system in expressing clinical therapeutic proteins is the absence of endotoxin (Gorbet and Sefton 2005). In addition, integrated expression is an attractive property of the P. pastoris expression system. The gene expression vector is linearized in vitro and then transferred into yeast competent cells. The open reading frame would be integrated into the yeast genome to achieve stable inheritance, thereby avoiding the loss of free expression plasmid, and saving the cost of using antibiotics (Figure 4).



(a)
Adaptation Modification
Expression Box Repetition
BOX
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AOX1
MF-alpha-1
gene
AmpR
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Recombinant Plasmid
Ori
AOX1 3'
PpHIS4
AOX1 Terminator
pPIC9K Vector
Linearization
Vaculoe
Mitochondria
Bud
Glycogen
Linearized plasmid
Golgi
Collagen
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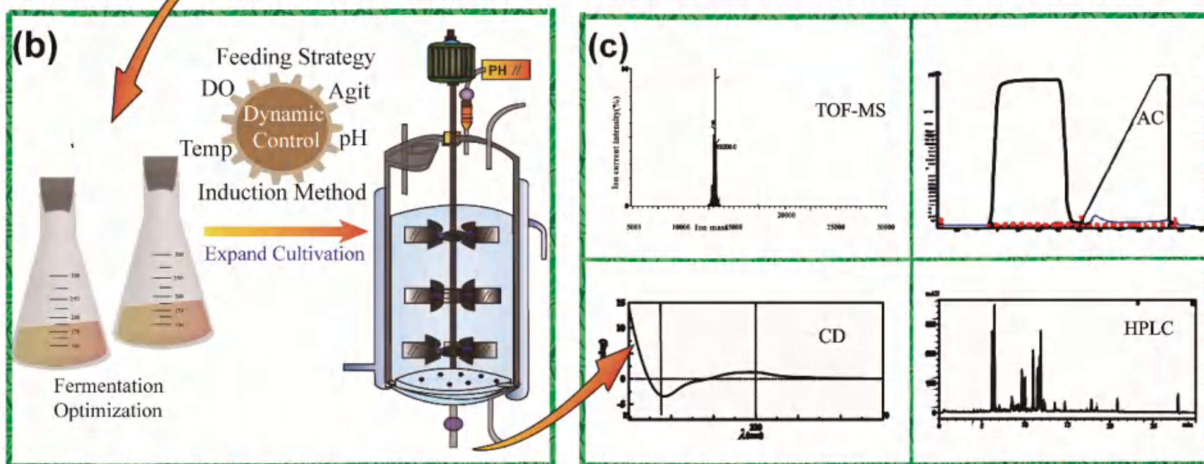

(b)
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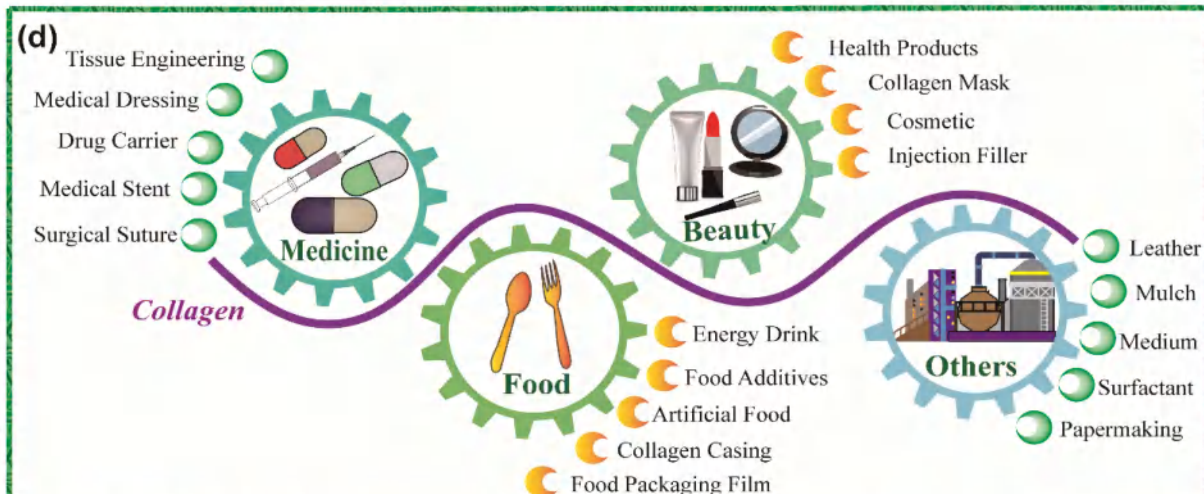


(d)
Tissue Engineering
Medical Dressing
Drug Carrier
Medical Stent
Surgical Suture
Medicine
Collagen
Health Products
Collagen Mask
Cosmetic
Injection Filler
Beauty
Food
Energy Drink
Food Additives
Artificial Food
Collagen Casing
Food Packaging Film
Others
Leather
Mulch
Medium
Surfactant
Papermaking

Figure 4 . Large-scale preparation and characterization of collagen in P. pastoris system. (a) The recombinant plasmid is linearized and transformed into yeast, then integrated into the chassis cell genome to achieve a stable heritage. (b) Recombinant strains are expressed in shake flasks and expanded cultivation in bioreactors. (c) Characterization and purification of fermentation products by AC, TOF-MS, CD, HPLC, etc. (d) Application of collagen in medicine, food, beauty and other industries.

Up to now, P. pastoris has exhibited relatively high expression quantity and hydroxylation efficiency in expressing collagen in recent years. For example, Wang et al. (2014) constructed a recombinant P. pastoris GS115/pPIC9K-COL3A1, the yield of human type III collagen reached 1.27 g L^{-1} via fermentation. In 2018, a report on the co-expression of human collagen gene and insulin gene (INS-COL1A1) in the P. pastoris system observed the yield of 300 mg L^{-1} at the shake flask level. In this case, the authors separately compared the pH, thermal, and enzymatic stability of the fusion protein INS-COL1A1 with the unfused protein recombinant human collagen α1 (I) chains (rhCOL1A1), and all the test results showed that the fusion protein INS-COL1A1 is more stable than rhCOL1A1, which was the first study that the use of insulin as a fusion protein could improve the stability of collagen (Mi et al. 2018). Previously, researchers combined reverse transcription and site-directed mutagenesis technology to construct gene expression vectors, and successfully expressed human collagen in the P. pastoris system, which achieved a high yield about 3.36 g L^{-1} (Li et al. 2015). These reports indicate that P. pastoris system plays an extremely important role in the production of collagen.

Recombinant expression in animal and plant cells for collagen production

Some researchers have attempted to express collagen in animal and plant cells. Using silkworms as hosts, Adachi et al. (2010) introduced the baculovirus trans-activator IE1 gene and the collagen gene into silkworm

cells, and the results showed that the content of recombinant collagen accounted for 8% of the cocoon. The products expressed by transgenic silkworms has not only fibroin but also collagens, which can be individually taken in different requirements to achieve killing two birds with one stone. Importantly, fibroin has more superior performance due to the introduction of collagen fiber. Besides, the use of corn as an expression host has also been reported (Xu et al. 2011). These animal and plant expression systems have their unique advantages, which can not only obtain the target protein, but also stimulate its products to be widely used in some hosts due to the introduction of collagen, such as artificial foods. The low expression level in animal and plant cells and the high cost of cell culture medium limit its application in commercial production. Therefore, future development of animal and plant expression systems, while promising, still requires striking the dual conundrum of yield and cost. For instance, Stein et al. (2009) co-expressed the human lysyl hydroxylase gene, prolyl hydroxylase gene, and type I human collagen gene in tobacco plants, and the content of Hyp and Hyl reached 7.55% and 0.74% respectively, which are similar to natural type I human collagen. The yield in this work was up to 20 g L^{-1}, which could meet the requirements of commercial production. Notably, this technique has been utilized by CollPlant Biotechnologies Ltd. for commercial production of recombinant heterotrimeric collagen type I (rhCOL1).

Molecular manipulation strategies to promote collagen production

Due to the large molecular weight of collagen and the fact that its gene sequence contains a large number of repeat sequences, amino acids such as Gly and Pro appear frequently. Besides, collagen also has strict post-translational modification effects such as hydroxylation and glycosylation, etc., which makes recombinant expression of collagen more difficult and

the expression level is limited. According to the previous work experience of predecessors, there are many molecular manipulation strategies worth trying to improve the expression of collagen. The adaptation modification of common elements such as RBS, promoter, signal peptide and so on (He, Sakaguchi, and Suzuki 2012; Hou et al. 2019; Püllmann and Weissenborn 2021), as well as co-expressed with fusion tags have been reported in other proteins. Through a large number of screenings of endogenous and exogenous elements of species, there is no lack of effective combinations. Unfortunately, those adaptation transformations and fusion expressions are rare in the expression of collagen, so, it is worth exploring in the future to improve the collagen yield. Of course, systematic screening of advantageous hosts and expression vectors with certain potential and codon optimization of gene sequences are also effective strategies to alter collagen expression levels radically. There has been a gene manipulation approach to increase the expression of interest protein by artificially raising the gene copy number in the P. pastoris expression system that attracted my attention. By repeatedly constructing the entire expression box from the promoter to the terminator, the copy number of the target gene was increased, thereby improving the translation level of the target protein (Zheng, Guo, and Zhou 2014). This method is theoretically applicable to different proteins, so it is also a good choice for the expression of collagen.

Protein expression is a very complex synthesis process, which involves several steps such as transcription, translation, folding, modification, and transport, as well as the regulation of thousands of regulatory factors. In the process of rapid synthesis, the folding flux of the endoplasmic reticulum may not be enough to meet the correct folding of all already translated proteins (Khan and Schroder 2008). The accumulation of unfolded and misfolded proteins would cause the unfolded protein reaction (UPR), which initiates the protein degradation pathway and affects the production of the active target protein (Read and Schroder 2021). Overexpression of

molecular chaperones and key enzymes related to folding in the endoplasmic reticulum can effectively promote folding and increase the expression level of recombinant protein, which is also a focus in current research. For example, heavy chain binding protein (Bip), has the function of instructing the folding, assembly, and direct misfolded proteins toward degradation pathways. Protein disulfide isomerase (PDI) can not only promote the formation of disulfide bonds in new peptide chains but also help to fold into a correct natural conformation in the endoplasmic reticulum. Yang et al. (2016) found that co-expression of PDI protein can significantly promote the secretory expression of porcine peptidoglycan recognition proteins (PGLYRPs) in P. pastoris system, while Bip made almost no contribution, suggesting that the co-expression of multiple types of the molecular chaperone should be explored according to the specific expression situation in the practical application process, which is not achieved overnight. In addition, secretory proteins, need to be transported to the extracellular through the secretory pathway after completing the post-modification in the Golgi apparatus. In the process of secretory expression, the deficiency of secretory flux also becomes a limiting factor for efficient expression. The co-expression of Sly1p, Sec1p, Sso1p, and other molecular chaperones related to protein transport can also play a critical role in promoting the protein yield (Hou et al. 2012). The application of these modification strategies to collagen production is indeed worthy to be attempted.

In short, the operation at the molecular level has great potential for enhancing collagen production, and different efficient novel strategies will be increasingly used in the efficient expression of collagen.

Fermentation strategies for collagen bioproduction

Production of collagen in E. coli

In order to realize the commercial scale production of foreign proteins, it is necessary to organically integrating the genetic engineering strategies with the large-scale fermentation technology. The E. coli system has been applied for the production of more than 400 recombinant pharmaceutical proteins (Ramshaw, Werkmeister, and Glattauer 2019). A suitable fermentation strategy is a key to achieving a high yield of recombinant collagen. Fruchtl, Sakon, and Beitle (2016) employed a fed-batch fermentation strategy to produce collagen binding domain fusion proteins by recombinant E. coli in a 3 L Applikon bioreactor equipped with Bioxpert Advisory software. Using glycerol as the initial carbon source and lactose at a concentration of 10 g L^{-1} as an inducer, the protein was well expressed by controlling the feed flow rate within 0.3 mL min^{-1}, pH at 6.8, the temperature at 37 °C, and controlling the agitator speed and ventilation rate to maintain DO values above 40%. After 12 h of automatic induction, despite the low cell density and only 35 g L^{-1} dry cell weight (DCW), the content of the obtained target protein per gram DCW was the highest, up to 20.2 mg g^{-1}. In the batch feeding process, carbon source and nitrogen source are crucial limiting factors, and it is necessary to reasonably control the timing and amount of feeding, as well as the dissolved oxygen, temperature, pH, and other indicators in the fermentation process. For example, when the dissolved oxygen does not reach the threshold conditions specified in the fermentation process, the growth of the bacteria will be restricted, and the fermentation state will be more severely affected or even irreversibly affected. During the fermentation process, the temperature control also has an important influence on the fermentation yield. Tang et al. (2016) cultivated recombinant E. coli in MBL medium to the production of hydroxylated human-like collagen in 10 L bench-top fermentor for scaled-up production, controlled the pH at an optimum value of 7.0, maintained more than 10% dissolved oxygen, and initiated induction with 0.1 mM IPTG for 13 ~ 15 h when OD_{600} was 25. Glucose was fed into

the broth to control the concentration at about 2 g L^{-1}, a two-stage temperature control strategy (37 °C for culture, 28 °C for induction) was used in the entire procedure and finally obtained 0.26 g L^{-1} yield.

Continuous optimization of fermentation conditions and investigation of various fermentation strategies is an effective method for improving collagen production. Peng et al. (2012) expressed the gene of bacterial collagen SCL2 with pColdIII vector in E. coli for fermentation production of collagen. It was firstly cultured at 37 °C for 24 h in a shake flask, then cooled to 25 °C and induced with 1 mM IPTG for 10 h. The yield of target protein at the shake flask level was about 0.2 ~ 0.3 g L^{-1}. To improve the output, the authors transferred from the shaker flask to a 2 L stirred tank bioreactors connected to the Biostat B control system for high-density fermentation. The yield of SCL2 was significantly increased to 19.3 g L^{-1} in defined medium (DM) at pH 7.0 by controlling the feeding rate of 15 mL h^{-1}, the agitator speed ranged from 500 to 1200 rpm, the airflow (containing 5% pure O_2) ranged from 0.3 to 15 L min^{-1}, and maintaining the DO value above 20%. It would lay the foundation for the high-yield fermentation of other types of collagens. In this study, the increase of collagen production was undoubtedly related closely to high-density fermentation. It is speculated that the proper fed-batch fermentation process greatly increases the biomass, which in turn increases the collagen production. In addition, in order to stimulate the specific "cold shock selectivity" of the pCold vector, the three-step cooling induction strategy (25 °C-10 h, 20 °C-5 h, 15 °C-9 h) has substantial contributed to the increase in the final yield.

Production of collagen in P . pastoris

Due to the unique advantages of yeast, there are many studies on the production of collagen by P. pastoris fermentation. Generally, the fermentation strategy adopted by P. pastoris is divided into two stages: the first stage is a batch fermentation generate sufficient biomass; the second

stage is a fed-batch fermentation, in which the yeast strain starves for a certain period and begin to be fed with methanol to realize high product accumulation (Azadi et al. 2017; Yang and Zhang 2018). For the PAOX1 system, the methanol feeding mode and rate of methanol are critical to the fermentation yield of the target protein because of the dual role of methanol as a carbon source and inducer in the induction phase (Yang and Zhang 2018).

Li et al. (2015) fermented human type III collagen by using the P. pastoris system in 250 mL shake flasks. Methanol solution was added to the culture liquid every 24 h, and the culture parameters were optimized by single factor experiment. And the results showed that the optimum production was obtained under the condition of continuous induction with 1% methanol for 72 h at 28.5 °C. Then, they switched to a 30 L mechanical stirring fermentor to further enhance the fermentation output. After 1 h of starvation, induction began at the condition of 28.5 °C and pH 5.0. As the oxygen consumption increases, adjust the stirring rate to 900 rpm gradually and control the intermittent period of methanol feeding for 60-sec to keep the DO above 30%, the yield reached 4.68 g L^{-1} by adopting the strategy of intermittent methanol feeding. This study provides an effective technical approach to produce collagen. In addition, the reduction of fermentation time will also have a great impact on the economic effect in industrial production. Wang et al. (2014) conducted the mixed fermentation of recombinant P. pastoris GS115 with 1% methanol and 8% glycerin in shaking flask, and the maximum collagen yield reached 1.27 g L^{-1}. Moreover, compared with single carbon induction, the fermentation time of mixed carbon induction fermentation strategy was shortened by 50%, which would greatly enhance the economic benefits of the product in subsequent large-scale synthesis.

However, excessive accumulation of methanol in cells might lead to cytotoxicity. It has been reported that methanol concentration higher than

3.6% would inhibit the growth of yeast and even lead to microbial death (Baghban et al. 2019; Ruottinen et al. 2008). Therefore, in the process of feeding, the feeding rate and feeding timing should consider not only the influence of limiting factors including temperature, dissolved oxygen, and inducer, but also focus on the residual concentration of each substance in the bioreactor, especially the methanol content. On the other hand, the production cost might also be taken into consideration in the feeding process. Furthermore, methanol is a flammable liquid, and the storage of a large amount of methanol would bring security risks for large-scale production. Therefore, the scientists began to seek alternatives for methanol. Recently, Wang et al. (2017) developed a methanol-free, P_{AOX1}-based P. pastoris strain (MF1) by knockout of three transcriptional inhibitors Mig1, Mig2, and Nrg1 and overexpression of transcriptional activator MIT1, and designed a "glucose-glycerol-shift" fermentation strategy to produce insulin precursor (IP). Subsequently, the authors delved into the high-density fermentation technology of novel engineered strain MF1 in a 5 L stirred tank bioreactor. In the glucose growth phase, the feeding rate of glucose (50%) was 50 mL h^{-1} L^{-1}. When the wet cell weight (WCW) reached approximately 200 g L^{-1}, the glucose growth phase was completed and then switched to the glycerol addition interval, in which 50% glycerol was added at a rate of 15.5 mL h^{-1} L^{-1}. During the induction phase, the temperature and pH were kept at 30 °C and 3.5, respectively, and the DO value was controlled above 30%. Ultimately, IP production reached 2.46 g L^{-1}, and oxygen requirement was significantly reduced. This study not only avoided the use of methanol but also reduced the consumption of oxygen and decreased the production costs, therefore this system becomes a promising methanol-free fermentation production system. However, the application of the methanol-free system for collagen production has not been reported, which could be attempted in the future.

Modification of collagen

The defects of natural collagen including poor elasticity, low mechanical strength, poor thermal stability, and easy degradation limited its specific application requirements. Collagen modification refers to changing the properties of collagen by certain strategies to strengthen stability, biocompatibility, or some other properties of collagen. In general, the modification methods of collagen can be divided into crosslinking modification and blending modification. The essence of collagen modification is to properly change the structure of collagen, and thus to modify its physical and chemical properties as well as improve its macroscopic performance. Table 2 lists the relevant cases of collagen modification in recent years.

Table 2. Cases of collagen modification in recent years. (Table view)

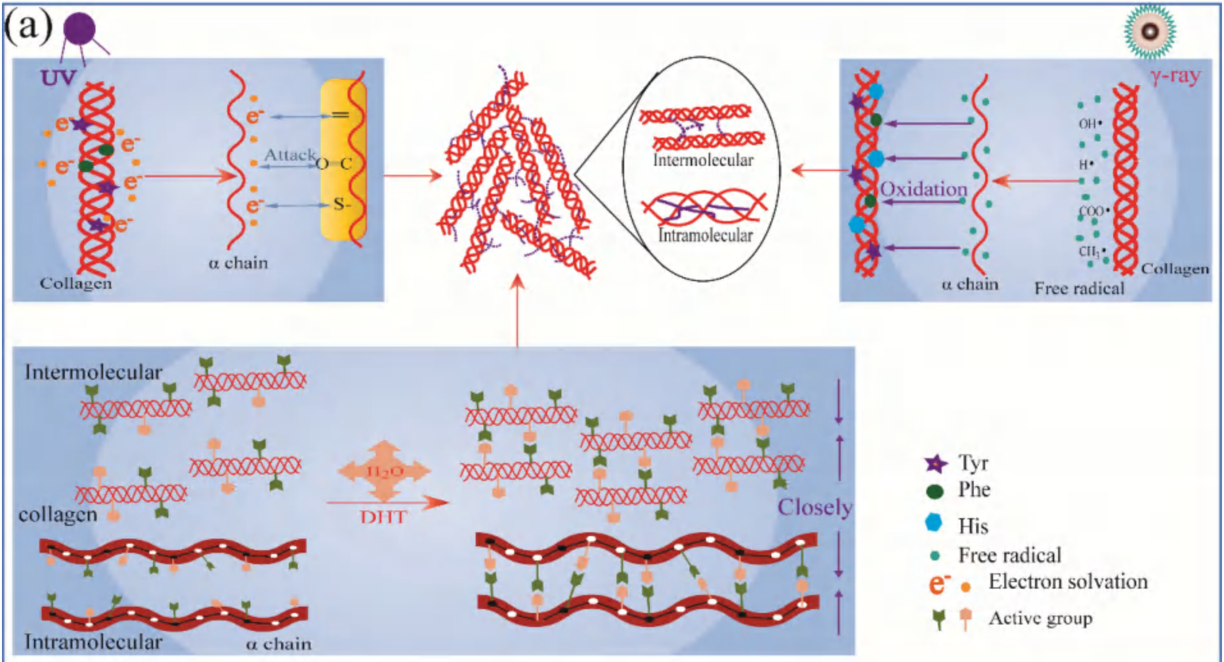
Cases	Modification methods	Cross-linker/ Enzyme	Measures	Improvements	Ap
Collagen fibers (Castro Santana, Kawazoe Sato, and da Cunha 2012)	Physical cross-linking	N/A	DHT	Viscosity decreased; Emulsification reduced.	Foo Indu
Collagen casings (Wang, Zhang, et al. 2015)	Physical cross-linking	N/A	UV irradiation.	Swelling resistance improved; Tensile strength increased; Thermal stability increased.	Foo Indu
Wet-spun collagen silk (Tonndorf et al. 2018)	Physical cross-linking	RF	UV irradiation, collagen cross-links with RF.	Cell compatibility increased; Thermostability enhanced; Ameliorated rigidity; Nontoxic.	Bio field

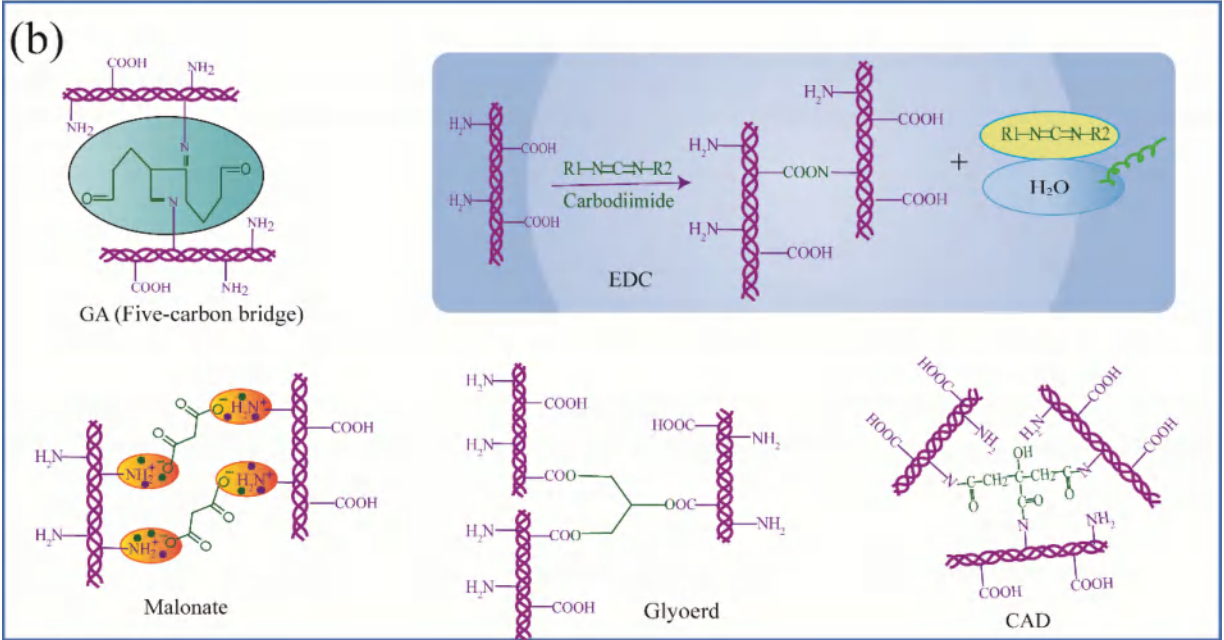
Cases	Modification methods	Cross-linker/ Enzyme	Measures	Improvements	Ap
ADA/CTS/collagen sponge (Du et al. 2016)	Chemical crosslinking	ADA, CTS	ADA, CTS and collagen cross-linking, lyophilized.	Structure tighter; Thermal stability increased.	Tiss eng
GA/collagen casing (Chen et al. 2020)	Chemical crosslinking	GA	Collagen cross-links with GA, drying to constant weight at 55 °C.	Low temperature thermal stability increased.	Foc Indu
Collagen fiber membrane (Cheng et al. 2019)	Biological crosslinking	Glutamine transaminase (TGase)	Enzymatic treatment.	Film thickness reduced; Mechanical properties increased; Thermal stability increased.	Foc Indu
SA/CTS/collagen sponge (Valenzuela-Rojo et al. 2020)	Blend modification	Sodium alginate (SA), CTS	SA/CTS/Collagen Combined, lyophilized.	Mechanical properties enhanced; Antibacterial properties improved.	Bio fielc
HA/collagen gel (Frayssinet et al. 2020)	Blend modification	Hyaluronic acid (HA)	HA and collagen blend crosslinking.	Low mechanical properties; Anti-enzymatic degradation; Highly hydrated.	Tiss eng
CMC-glycerin-collagen composite membrane (Wu et al. 2020)	Blend modification	Carboxymethyl cellulose (CMC)	CMC, glycerol and collagen blend crosslinking.	Tensile strength increased; Thermal stability enhanced.	Foc Indu

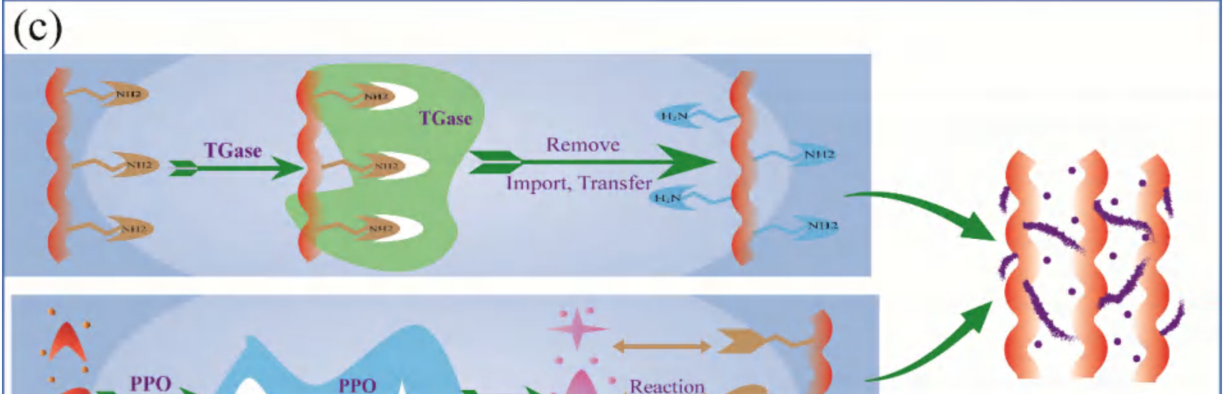
Cases	Modification methods	Cross-linker/ Enzyme	Measures	Improvements	Ap
sHA3/collagen scaffold (Al-Maawi et al. 2022)	Blend modification	High-sulfated hyaluronan (sHA3)	Covalent bonding of sHA3 to collagen fibrils.	Physical stability enhanced; Control growth factors released; Inflammatory reaction reduced; Non-multinucleated giant cells.	Bio fiel

Cross-linking modification of collagen

Crosslinking modification means that the tension and stability of collagen fibers could be improved by intramolecular and intermolecular covalent binding. The cross-linking method can be divided into physical, chemical, and biological cross-linking (Figure 5). Each method has its own advantages and disadvantages (Liu et al. 2019).


(a)
UV
Attack
α chain
Collagen
Intermolecular
Intramolecular
Oxidation
γ-ray
Free radical
DHT
Closely
Tyr
Phe
His
Free radical
Electron solvation
Active group
collagen


(b)
GA (Five-carbon bridge)
Carbodiimide
EDC
H2O
Malonate
Glyoerd
CAD


(c)
TGase
Remove
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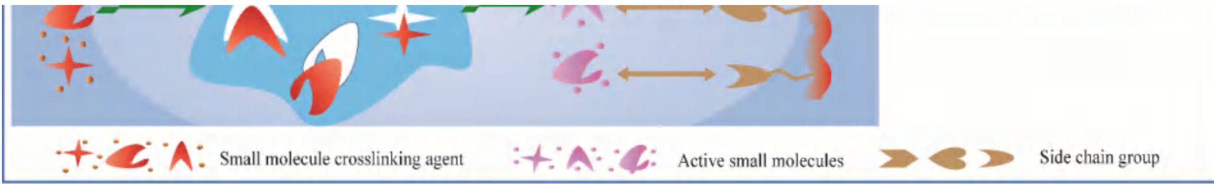

Figure 5. Physical, chemical, and biological cross-linking modification of collagen. (a) Physical cross-linking. UV-rays are absorbed by Tyr and Phe in collagen and photoionized to form solvated electrons, then react with double bond groups and free sulfhydryl groups to form intermolecular and intramolecular covalent crosslinks; *γ*-ray radiation uses free radicals generated by high-energy rays to oxidize Phe, Tyr and His in collagen, and the oxidation products react with adjacent amino acids to form stable covalent cross-links; DHT can shorten the distance between the active groups. (b) Chemical cross-linking. GA forms a five-carbon bridge with collagen; EDC promotes crosslinking and is removed with water; non-covalent bond between malonic acid and amino group of collagens. (c) Biological crosslinking. TGase catalyzes the amino groups on the side chains and PPO catalyzes the small molecule cross-linking agent to realize molecular cross-linking.

Physical crosslinking

The physical crosslinking-based modification could be performed via ultraviolet (UV) irradiation, γ radiation, and dehydration heat treatment (DHT). After UV treatment, UV is absorbed by aromatic amino acids (Tyr, Phe) in collagen and photoionized to form solvated electrons, which can react with double bond groups and free sulfhydryl groups to form intermolecular and intramolecular covalent cross-linking (Gennadios et al. 2008). After proper UV treatment, the expansion resistance, tensile strength, and thermal stability of collagen casing membrane are improved to a certain extent (Wang, Zhang, et al. 2015). However, the content of aromatic amino acids and cysteine in collagen is relatively low, thus the degree of cross-linking by UV is poor. Radiation of γ-ray employs free radicals generated by high-energy rays to oxidize Phe, Tyr, and His in collagen. The oxidation products react with adjacent amino acids to form stable covalent cross-links (Koshimizu et al. 2009). The DHT causes the cross-linking of collagen molecules as it can shorten the distance between the active groups. After treatment, the denaturation temperature and physical properties of collagen could be improved. Compared with

chemical crosslinking, physical crosslinking methods generally improve the mechanical and physical properties without toxicity or residual crosslinking agent; however, it was reported that collagen would degrade under long-term exposure to physical stimulation. In the process of exploring the effect of heat treatment on the emulsifiability of collagen fibers, the viscosity and emulsifying property of collagen fibers decreased after heat treatment at 50 ~ 85 °C for 20 or 60 min (Castro Santana, Kawazoe Sato, and da Cunha 2012). It is difficult to obtain collagen materials with high strength and uniform crosslinking by physical treatment alone, and other strategies could be considered in combination with physical stimulation (Kozłowska and Sionkowska 2015; Liu et al. 2019). According to the study of Tonndorf et al. (2018), they attempted to investigate whether the riboflavin (RF)-induced light crosslinking method could replace the chemical crosslinking method with glutaraldehyde (GA) as a crosslinker to prepare wet spun collagen fillings. Their results found that the combination of RF and UV irradiation could crosslink collagen to form wet spun collagen filaments (Coll + RF + UV) without cytotoxicity. The mechanical properties of Coll + RF + UV were increased (the highest tensile strength: 1 cN tex^{-1}, the modulus: 1 dN tex^{-1}), and at the same time, the thermal stability had a further improve (the denaturation temperature: 55 °C, the denaturation enthalpy: 29 J g^{-1}) compared with the non-crosslinked wet spun collagen filaments (Coll); hMSC cells were cultured in wet spun collagen filaments crosslinked with GA (Coll + GA) and Coll + RF + UV culture medium to assess the cell response to the two types of collagen filaments, and the number of cells in Coll + RF + UV reached 7300, but only 3500 in Coll + GA after 14 days. Coll + RF + UV showed higher high cell compatibility than Coll + GA.

Chemical crosslinking

Chemical cross-linking is currently the most commonly used cross-linking modification method, which mainly utilizes chemical reagents to react with the carboxyl or amino groups of collagens to change the composition of reactive groups. Due to the high cross-linking degree, uniform cross-linking, and strong control between collagen and aldehydes, aldehydes are generally used as the traditional chemical modification materials of collagen. The structure would be more compact, and meanwhile, the thermal stability was significantly improved via adding alginate dialdehyde (ADA) into COLL/CTS sponge, and also ADA had no effect on the antibacterial activity of COLL/CTS material (Liu et al. 2017). Among all aldehydes, GA shows a favorable cross-linking effect with collagen. The two aldehyde groups of GA can react with the primary amino group in collagen to form a Schiff base, which connects collagen with a five-carbon bridge structure. And the reaction is irreversible. Cross-linking of GA and collagen at a relatively low drying temperature (55 °C) can improve the thermal stability of collagen under low-temperature (Tian et al. 2014). GA is one of the most commonly used crosslinking agents in biomedical materials (Cheng et al. 2019), but it has been proven to be potentially cytotoxic and should be used with caution (Valenzuela-Rojo et al. 2020).

In order to avoid the cytotoxicity of GA, other cross-linking material candidates have been under exploration. The cytotoxicity of glycerol is negligible, and the three hydroxyl groups are esterified with the carboxyl groups on the side chain to form a three-dimensional spatial network structure. The three active esters of citric acid derivatives (CAD) can react with the amino group of Lys or Hyp to form an amide bond, and its toxicity is greatly reduced, which is only 1/10 of that of GA (Taguchi et al. 2004). In addition, carbodiimide (EDC), as an activator of a carboxyl group, can promote the carboxyl group of Glu or Asp to react with amino group to form an amide bond. Although EDC itself is cytotoxic, its crosslinked form would be generated to produce water-soluble urea derivatives, which are

finally eliminated with water. It is also a promising strategy to avoid the introduction of toxic agents.

Most of the commonly used chemical crosslinking agents are covalent in the crosslinking process, while dicarboxylic acids can interact with collagen through non-covalent bonds. The carboxyl group of malonic acid (low toxicity) and amino group of Lys form a new three-dimensional scaffold through proton exchange and ion interaction, which can improve the mechanical properties and thermal stability of modified collagen. According to a project that prepared composite scaffolds using malonic acid modified collagen, its reported that the mechanical properties performed best when the concentration of malonic acid was 0.2%, with a tensile strength of 7.85 MPa, an elongation at break of 11.8%, a stiffness of 1.32 N mm^{-1}, a Young's modulus of 65.97 MPa, and an extension at maximum load of 3.14 N. At this point, the crosslinking degree is 78% with considerable thermal stability, and the degeneration temperature rises to 128 °C (Mitra et al. 2012).

Although a variety of chemical cross-linking agents have been developed, scientists are always moving forward to find low-toxic, nontoxic chemical cross-linking agents and new alternative strategies.

Biological crosslinking

Biological crosslinking mainly induces collagen to produce new crosslinks by enzymatic reaction. Representative enzymes include TGase and polyphenol oxidase (PPO). TGase catalyzes the removal, introduction, and transfer of amino groups in the side chain of protein molecules to achieve covalent crosslinking. While PPO catalyzes small molecule crosslinker to form active groups which are easy to crosslink with side chain groups, so as to realize the intramolecular and intermolecular cross-linking of collagen through the bridging of the active groups. TGase has been used to catalyze the crosslinking between glutamine and lysine residues to modify the

structure of proteins. Bio-crosslinking modification of collagen film by TGase, the thickness of collagen film decreases, but it significantly improved mechanical properties and thermal stability (Cheng et al. 2019). Biological crosslinking shows a promising prospect in collagen modification due to the high efficiency and low toxicity, but the moderate crosslinking efficiency also limits its wide application.

Blending modification of collagen

Blending modification is a modification strategy that blends collagen with other natural or synthetic polymer materials to form a kind of multi-phase solid material, which could effectively improve the application performance of collagen materials. In recent years, the blending modification of collagen and natural polymer materials with favorable biocompatibility, such as CMC, CTS, chondroitin sulfate, HA, heparin, fibroin, etc., has attracted substantial attention. Combination of CTS and sodium alginate with collagen for preparing medical dressing antibacterial collagen sponge shows good mechanical properties (Valenzuela-Rojo et al. 2020). Collagen and HA could be used to produce biomimetic hydrogels with high resistance to enzymatic degradation, high hydration, and increased survival level and attachment status of cells, which has enormous potential in tissue engineering applications (Frayssinet et al. 2020). Wu et al. (2020) prepared a collagen fiber-glycerol-CMC composite membrane via adding CMC and glycerin aqueous solution to the collagen fiber paste with the impregnation method. The characterization results indicated that the interaction between CMC and collagen leads to a dense film structure, and the mechanical properties and thermal stability of the dry film are improved. Compared with composite membranes without CMC, the young's modulus and maximum tensile strength (dry membranes) of collagen fiber-glycerin (4%)-CMC composite membranes increased by 2.77% (1.58-5.96 MPa) and 125% (21.49-48.49 MPa) respectively, thermal shrinkage values decreased

by 10 percentage points (51.7%-41.7%) as the content of CMC increased from 0 to 1.5%. And also, when the content of CMC increased from 0.5% to 1.5%, its denaturation temperature was increased from 98.40 °C to 105.58 °C. Although collagen can improve related properties after modification, the strong inflammatory response that may be caused is also an important indicator that we need to consider. In a recent study, Al-Maawi and coworkers (2022) evaluated a novel type of scaffold-sHA3 + EDC/NHS prepared by covalent conjugation of high-sulfated hyaluronic acid (sHA3) to a collagen scaffold (Mucograft®) that has been used in the clinic, which not only has the physical stability of collagen structure, but also shows considerable ability to control the release of growth factors in vitro. In particular, it effectively reduces the inflammatory response caused by proinflammatory macrophages, and does not form multinucleated giant cells in vivo. In the future, the blending modification of collagen with synthetic polymer or natural polymer would always be a hot research topic in order to meet the application demands.

Practical application of collagen

Food field

Due to the absence of tryptophan in gelatin and the very small or even close to zero content of cysteine, it has been considered as incomplete protein and has no nutritional value for a long time. Until the concept of "collagen" was put forward and elaborated that collagen contains 7 kinds of essential amino acids and more than a dozen non-essential amino acids, as well as extremely rich Hyp, which fundamentally reversed this perception and made the collagen become widely used in the food industry (Figure 4).

Collagen is often utilized as an emulsifier, foaming agent, stabilizer, clarifier, and other food additives due to its excellent physical properties in the food industry (Bhuimbar, Bhagwat, and Dandge 2019). Collagen has an amphiphilic structure with both hydrophilic and hydrophobic regions,

which could be used as a natural emulsifier (Pires and Batista 2013). For instance, margarine is one of the most representative collagen emulsifying products. Collagen also plays a crucial role as the stabilizing agent in food. Collagen acts as both emulsifier and stabilizer in margarine. In the process of food freezing, antifreeze peptides in collagen hydrolysates could control or prevent the formation of ice crystals and delay the melting rate in the cryopreservation of food (Wang, Chen, et al. 2015). The studies of Yang et al. (2009) also revealed that the activities of retorted skin gelatin hydrolysates (RSCH) could improve the stability by reducing the oxidation activity of food. This study revealed that RSCH not only has 79.4% scavenging ability for α, α-diphenyl-*β*-picrylhydrazyl (DPPH), but also has a noticeable effect on suppressing lipid peroxidation by 77.1%, which could effectively delay lipid deterioration and act as a protective agent for oil products. And the authors found that the antioxidant activity of hydrolysates with molecular weight lower than 6500 Da was higher, and speculated that some amino acid residues and oligopeptides in RSCH played an antioxidant role. Collagen molecule is amphoteric electrolyte in aqueous solution, which can form flocculent precipitation with tannin and other acidic substances. After filtration and removal of precipitation, it could make drinks such as wine and fruit juice more clear and better taste, thus it is usually used as beverage clarifier. The collagen hydrolysate clarifier prepared with pigskin shavings by Zhang et al. (2018) showed an excellent clarification effect in the study of chrysanthemum beverage, the transmittance was increased by 40%. Furthermore, the overall sensory quality was increased from 62.7 to 85.6, and the storage time was extended from 1 day to 12 days, indicating that the stability was also significantly improved. Nowadays, several beverages on the market employ collagen as the food additives to improve the nutrition, taste, viscosity, flavor and other characteristics of the product (Bilek and Bayram 2015; Gerhardt et al. 2013; Rigoto et al. 2019).

Since researchers in food field are looking for various food packaging films with antibacterial activity, collagen display promising film forming property, which could be used not only as medical film but also as food packaging film (Bhuimbar, Bhagwat, and Dandge 2019; Jiang et al. 2020; Wang and Rhim 2015). It is an inevitable trend that plastic food packaging is replaced by green packaging (including edible and degradable packaging), especially edible packaging, which are getting much attention in the market. Before the 1930s, humans used natural casings for processing sausages, such as sheep casings (Chen et al. 2019) and pig casings. Natural casings are considered to be the gold standard for sausage casings due to their edible, cooking resistance, air permeability, and flexibility (Suurs and Barbut 2020). However, the high cost and limited supply of natural casing made it difficult to meet the market demand; therefore, the United States and the United Kingdom began to study collagen-based casings in the 1930s. In the 1960s, the first artificial collagen-casings came to market, ushering in the modern sausage era (Suurs and Barbut 2020). As casings are prone to rupture during cooking or enema (Adzaly et al. 2016), poor cooking resistance and tensile properties are two major bottlenecks in the preparation of artificial collagen casings. Scientists are constantly trying to introduce cross-linking methods (physical cross-linking (Shi et al. 2019a; Wang, et al. 2020) and chemical cross-linking (Olde Damink et al. 1995; Weadock et al. 1996) to overcome this difficulty (Chen et al. 2020; Chen et al. 2019). Today, novel casings with superior performance are produced using a mass-produced co-extrusion technique that coats and gelatinizes sausages with a collagen-alginate mixture (Hilbig et al. 2019; Marcos et al. 2020; Suurs and Barbut 2020; Walz et al. 2018). Collagen has become one of the indispensable elements in the food industry and would have a broader application prospect for food processing.

Biomedical field

Collagen exhibits a series of favorable biological characteristics such as good affinity and biocompatibility, which has been widely used in the biomedical field (Figure 4). As early as more than 2000 years ago, collagen sutures began to form an indissoluble bond with ancient medicine (Ramshaw 2016), which opened the brilliant prelude of collagen in the field of medicine. Nowadays, collagen is in an indispensable position in life science community, and its application is continuously expanded. In 2015, Huang et al. (2015), for the first time, demonstrated that the collagen in fish scales has a binding activity with Fe (II). On this basis, scientists utilized human collagen to prepare a new iron agent (SH-HLC-Fe), which improved the absorption and utilization ratio of iron (Zhu et al. 2017). On the other hand, however, the natural collagen is unstable when the temperature is higher than 40 °C. Scientists employ other materials in combination with collagen to enhance its stability (Menezes et al. 2020). The recombinant collagen-HA (rCOL-HA) composite scaffold developed by He et al. (2020) showed noticeable water absorption (thirtyfold of own weight after absorbing water), thermostability (denaturation temperature of rCOL-10% HA: 98.2 °C), mechanical property (withstanding 2.5 N of tension), and high porosity (over 90%). And it was proven that the collagen-HA composite scaffold can meet the requirements for clinical application.

In literature, the main application forms of collagen are gels (Claudio-Rizo et al. 2020; Li et al. 2019), sponges (Alagha, Nourallah, and Hariri 2020), films (Andonegi et al. 2020; Walczak et al. 2020), biological scaffold (He et al. 2020; Yu et al. 2020), sheets (Alberti et al. 2014), injectable solutions (Moreira et al. 2016), etc. In recent years, their application areas are becoming more extensive, including antibacterial dressings (Shalaby et al. 2020), hemostatic materials (Sun et al. 2020), surgical sutures (Rethinam et al. 2018), tissue engineering (Gu et al. 2019; Miranda-Nieves and Chaikof 2017). In addition, collagen is also promising in the development of drug delivery systems (Liu et al. 2019; Subhan et al. 2021).

Seong et al. (2020) has developed a porous calcium phosphate-collagen composite microsphere for the delivery of growth factors during bone regeneration. Collagen fibers could increase the surface area inside the microspheres used for drug adhesion, which significantly improved the drug loading efficiency and promoted the bone growth. The common way to treat glaucoma in medicine is to administer drugs with eye drops frequently, the preparation of cross-linked collagen shield sustained-release drug delivery system by means of nanotechnology has opened up a new path for the treatment of this disease. Agban et al. (2016) applied polyvinylpyrrolidone capped zinc oxide nanoparticles (ZnO/PVP NPs) to cross-link collagen in a 1:1 ratio for delivery and extended release of Pilocarpine Hydrochloride (PHCl). By evaluating this collagen shield in terms of tensile strength, thickness, transparency, pH, as well as cytotoxicity, it was suitable for ocular applications, and sustained release of PHCl about 14 consecutive days, which demonstrated more sustained drug release capability than at least twice daily administration with eye drops.

In recent years, collagen has also begun to play a critical role in the tissue engineering materials area. Ge et al. (2018) successfully synthesized Collagen/DXG-AgNP composite dressing, which has the effect of promoting collagen deposition and exhibits obvious healing effect on full-layer deep burns without forming scars. Moreover, since the advent of 3D printing technology, the preparation of numerous collagen-based tissue engineering materials has been reported. Recently, Bavaresco et al. (2020) prepared a collagen-HA scaffold with the combination of 3D printing technology to promote cells adhesion and proliferation, which has potential in soft tissue engineering field. In addition, the application of collagen in blood vessels (Zhou et al. 2018), liver (Miranda-Nieves and Chaikof 2017), heart (Fu et al. 2017), skin (Greco et al. 2018), bone (De Melo Pereira, Davison, and Habibović 2022; Gilarska et al. 2020), cartilage (Irawan et al. 2018), tendon (Sawadkar et al. 2019) and other tissues and organs have also

been extensively concerned by researchers (Liu et al. 2019; Miranda-Nieves and Chaikof 2017). It is expected that collagen would be attracting increasing interest in the biomedical engineering field with the development of biotechnology and genetic engineering technology as well as activity-oriented collagen modification strategies.

Beauty field

The ability of fibroblasts in the body to synthesize collagen will gradually decrease with age. The phenomenon of body aging is closely related to the decrease of soluble collagen, which leads to the weakening of water retention capacity, the inability to swell, and the formation of inelastic connective tissue. Therefore, the supplement of collagen occupies a pivotal position in cosmetology (Figure 4).

Collagen has been proven harbor excellent moisturizing and water-locking behavior, which could be used as various cosmetics formulations (Alves et al. 2017). Hydrolyzed collagen is rich in hydrophilic groups (e.g., $-CONH_2$, $-COOH$, $-OH$) (Li et al. 2020), and glycine, alanine, serine, aspartate also are natural moisturizing factors. Small molecule collagen can be easily absorbed by skin dermis to improve skin moisture content, showing its potential as a moisturizing factor in the interior. While high molecular weight collagen could not be absorbed by skin corneum, it can combine moisture on the outer surface of skin through hydration to increase skin-humidity (Swatschek et al. 2002). The water retention capacity of the collagen in the plum-extracted sea cucumber is significantly higher than that of the glycerin control group (Li et al. 2020). The collagen-related products such as creams and lotions with moisturizing effects is extensively explored and developed in the cosmetics industry (Berardesca et al. 2009; Xhauflaire-Uhoda, Fontaine, and Pierard 2008).

The tyrosine residues rich in collagen could play a role of competitive inhibitor, and compete with tyrosine in the skin for the binding site of

tyrosinase active center, inhibit the production of melanin in the skin, which would be benefit for skin whitening (Aguirre-Cruz et al. 2020; Schurink et al. 2007). A clinical study evaluating a new oral supplement prepared by combining fish skin collagen with soy peptide has revealed its antioxidant and inhibitory effects on tyrosinase activity, resulting in reduced facial pigmentation, skin lightening, and whitening of patients (Gui et al. 2017). Hydrolyzed collagen can inhibit the activity of matrix metalloproteinases, exhibits positive effects of anti-oxidation (Kim et al. 2006; Venkatesan et al. 2017) and anti-aging (Czajka et al. 2018; Haydont, Bernard, and Fortunel 2019; Ngo et al. 2012; Wang et al. 2018), and also can ameliorate the drying, relaxation and wrinkles caused by skin aging. It is generally considered to be a safe and effective cosmetic material (Aguirre-Cruz et al. 2020).

In the field of beauty, antioxidants seem to be an inevitable topic. A bright research offers a more nuanced interpretation of the components responsible for the antioxidant effects of collagen recently. The collagenase gene extracted from Bacillus cereus was heterologous expressed in E.coli Rosetta (DE3), and the expressed product was used to hydrolyze bovine bone collagen protein to prepare collagen-soluble peptides (CPP). The enzymatic hydrolysis process was optimized by response surface methodology, and the CPP prepared by reaction for 6 h at an enzyme concentration of 110 μg mL^{-1} at pH 8 and 35 °C exhibited excellent antioxidant effect with a reducing power of 0.41, the scavenging rate of DPPH radical was 18.98%, and the rate as high as 99.21% for ABST radical. Five small peptides with the highest antioxidant activity and molecular weight of 600-900 Da were obtained by gel chromatography. The ABTS radical scavenging rate of CPP-3-a (GPIGPVGAR, 822.47 Da) reached 47.16% at the concentration of 5 mg mL^{-1}, and the highest DPPH radical scavenging activity was 47.51% generated by CPP-3-e (GQAGVMG, 618.28 Da). More importantly, it was found that these five

antioxidant peptides all had Gly-X-Y, a typical collagen repeat sequence structure (Song et al. 2021). This explained the speculation of Yang et al. (2009) that some amino acid residues and oligopeptides in RSCH had antioxidant effect, and it also proves that collagen does contribute significantly to the antioxidant effect.

Collagen is the nutrient base of the hair in the epidermis layer, providing nutrition and moisturizing for hair. Sionkowska et al. (2017) prepared Coll/CTS/HA biopolymer film for hair care with superior hydrophilicity and mechanical properties, and its high surface area increases the contact area with hair, which could effectively enhance the toughness, thickness, and beauty of hair.

In addition, after being absorbed by the skin, collagen would be filled between the skin matrix to make the skin plump and wrinkle stretched (Jhawar, Wang, and Saedi 2020; Lee et al. 2019; León-López et al. 2019; Sant'Anna Addor, Vieira, and Abreu Melo 2018). Injecting collagen into sunken skin or damaged tissue could induce adjacent fibroblast cells to gather at the damaged area and synthesize its own collagen, forming connective tissue and repairing skin (Lauer-Fields and Fields 2002; Lee et al. 2021). After the first batch of bovine collagen product Zyderm® was put into the market in 1976, the State Food and Drug Administration (FDA) formally approved it for clinical use in 1981, which was widely promoted in Europe and Japan (Athre 2007). In 1985, the FDA approved the clinical application of Zyplast®, a bovine collagen product cross-linked with GA, which could slow down the decomposition by collagenase, thus the half-life is longer than Zyderm®, and subsequently, human collagen products cross-linked with GA, such as Cosoplast®, also came out successively (Athre 2007; Noguera et al. 2004). Bovine collagen, along with HA and polyacrylamide, are the most commonly used filling materials (Beer 2009). Generally, the skin test is a necessary step before the injection of bovine collagen, and there have been cases of anaphylactic shock caused by the

injection of bovine collagen filling materials (Mullins, Richards, and Walker 1996; Siegle et al. 1984). To this end, researchers developed the human collagen implants to avoid allergic reactions. Autologen® has been proven to be able to effectively avoid allergic reactions. The supporting function of collagen is outstanding in maintaining human body curves and showing straight and upright posture. It has been applied in plastic surgery such as nose augmentation (Kaplan, Falces, and Tolleth 1983) and lip augmentation (Yamasaki and Lee 2020).

Conclusion and outlook

Collagen is the most abundant protein in the human body. In view of its unique structure and special properties, collagen has become one of the most widely investigated biomacromolecules to date. Since the production and performance of collagen obtained by traditional extraction methods are difficult to meet the market demand, the scientists employed genetic engineering technology and high-density fermentation method to produce collagen and modify the original collagen to enhance the application performance. In this review, the development advances and trends were demonstrated by introducing and summarizing the heterogenic expression, fermentation production, and modification of collagen in recent years, which laid the foundation for future research in this field.

Although tremendous progress on collagen preparation has been made in recent years, there are still many problems to be solved. Heterologous expression and high-density fermentation production of collagen exhibits significant advantages; however, it is still necessary to further improve its production level through gene engineering and optimization of fermentation strategies. Furthermore, the formation of inclusion bodies and endotoxin production during the expression in E. coli system are obstacles for commercial production and wide application. Chemical crosslinking is still the most widely used modification method at present, searching for new

alternative modification strategies still needs to be further attempted and explored. It is believed that the application of collagen in food, beauty, and biomedicine would be further expanded with the continuous progress of biotechnology and novel molecular modification technology as well as multidiscipline approaches.

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