

## Method for purification of collagen: A systematic review

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**Abstract.** Collagen has diverse applications in various industries and thus a various method of purifications has been developed over the years to produce high yield and high purity of collagen to fulfill collagen market demand. The objective of this qualitative systematic review is to summarize the current literature on the existing purification methods for collagen from different sources. Toward this end, three databases were searched and articles were screened for all original articles explaining the purification of collagen regardless of their main sources. After the comprehensive screening, out of 715 articles, 12 articles were chosen and were further reviewed. The analysis of the technical details, theory, advantages, and disadvantages of the techniques used are reported in this study. We found four types of purification methods that commonly used to purify various types of collagen from mammals, marine, bacteria, and recombinant collagen. The correlations between the prevalence of the method and the efficiency of collagen purification were also identified. It highlighted that many factors should be considered before choosing any method of purification such as types, sources and structure of the collagen itself. Affinity chromatography commonly used for purification of recombinant collagen as they have been genetically modified and appended by affinity tags whereas conventional purification method is preferable for non-recombinant collagen from mammalian and marine sources.

**Keywords:** collagen, purification, ATPS

## INTRODUCTION

Collagen is the most abundant protein in the extracellular matrix of the animal kingdom, including all vertebrates and invertebrates (Fidler *et al.*, 2018; Kadler *et al.*, 2007; Ricard-Blum & Ruggiero, 2005). About 30% of total proteins in mammals are represented by collagen. It is a fibrous structural protein that can be identified by containing three polypeptide chains that twisted together in the form of a triple helix structure (Berisio *et al.*, 2002; Hashim *et al.*, 2015; Kittiphattanabawon *et al.*, 2015; Swatschek *et al.*,

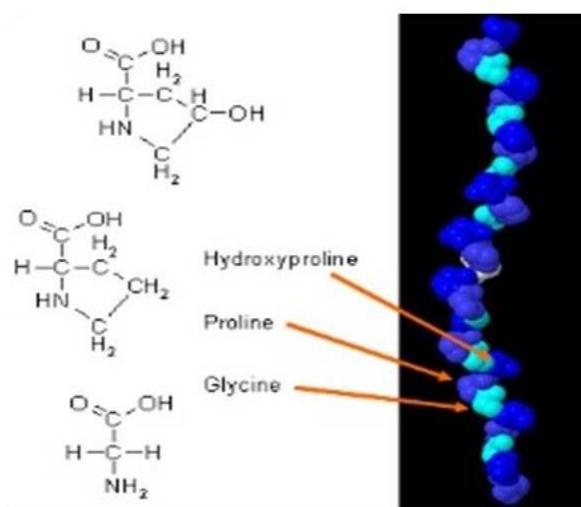
2002; Werkmeister & Ramshaw, 2015; Yamazaki *et al.*, 2010).

The special characteristic of the structure is the tight packing of the triple helix that requires a small amino acid which is glycine (Gly) to fill the interior axis of the structure. Hence, it leads to the formation of a repetitive sequence pattern (Gly-Xaa-Yaa)<sub>n</sub> for each polypeptide, which is a distinctive feature of collagens (Brodsky & Ramshaw, 1997; Muyonga *et al.*, 2004). In mammalian collagen, the Xaa and Yaa positions are usually represented by proline and

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hydroxyproline respectively (Werkmeister & Ramshaw, 2015). Hydroxyproline (Hyp) is the product from the post-translational modification of amino acid proline (Gorres & Raines, 2010). Figure 1 shows the amino acid structure in collagen molecules (Yamazaki *et al.*, 2010). Collagen plays a crucial role in the human body especially in maintaining elasticity, firmness, and strength to the skin and helps in tissue and organ development. Collagen also protects the skin by inhibiting the absorption of toxins and pathogens (Silvipriya *et al.*, 2015).



**Figure 1.** Triple structure helix of collagen (Silvipriya *et al.*, 2015).

In recent years, recovery and purification of proteins are the major challenges due to its complexity and high cost (Stitt & Weatherley, 1994). Purification process which is the most crucial step and makes up more than 70% of the downstream processing cost (Alves *et al.*, 2000). The conventional methods consist of several unit operations that lead to a high cost of operation and maintenance (Przybycien *et al.*, 2004; Raja *et al.*, 2012; Song *et al.*, 2013). For instance, precipitation needs to be combined with other processes such as chromatography processes, which are complex, tedious, time-consuming and often produced low yields (Cao & Xu, 2008; Sato *et al.*, 2003; Wang *et al.*, 2017). Furthermore, the high number of unit operations involved causes a high loss of target molecules that will result in low product yield (Xing *et al.*, 2012).

Applications of collagen emerged in various fields such as pharmaceutical, food, and cosmetic industry. Due to its biodegradability, cell

attachment ability, weak antigenicity, and biocompatibility, collagen showed high demand as microparticles, injectable dispersions, shield in ophthalmologysponges, and drug delivery system (Kaufman *et al.*, 1994; Liu *et al.*, 2010; Shin *et al.*, 2016). As humans getting older, the production of collagen getting slower results of losing the human skin elasticity. Thus, the most common ingredient in cosmetics especially in skin care products is collagen. Alike pharmaceutical industry, collagen has great potential in the food industry as a component for texturizing and thickening due to its good water absorption capacity (Felician *et al.*, 2018; Hashim *et al.*, 2015).

The objective of the present review is to summarize and view the current literature on the existing purification methods for collagen from various sources. The advantages and disadvantages of every method were carefully explored and the major outcomes of purification, yield, and purities were discussed.

## MATERIALS AND METHODS

The present methodology is in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.

### *Inclusion process*

The first step on this process was to make extensive literature search in Scopus (<http://www.scopus.com/>), ScienceDirect (<https://www.sciencedirect.com/>) and PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) databases that were published up until 9<sup>th</sup> of May 2019 using the following keywords: (collagen OR recombinant collagen) AND (purification OR isolation OR extraction). The selection of journals in this review emphasized the high impact journals that specialized in a particular field. Papers that did not report the method of purification of collagen, review articles, and articles that were written in foreign languages were excluded. Due to the limited studies on this topic, there were no limitations regarding the year of publication. No restrictions for the method of purification, sources of collagen, type of analysis, and quantification of results. The articles that reported the following data were included: 1) the

details of purification steps 2) sources of collagen 3) purity or yield of collagen produced.

### **Variables of interest**

The following data were extracted from the included publications: 1) the year of publication 2) sources of the collagen 3) method of purification/extraction 4) advantages of each method 5) disadvantages of each method 6) method of collagen quantification and 7) the product yield.

## **RESULTS AND DISCUSSION**

### **Inclusion Process**

The inclusion process is summarized in Figure 2. By using the establishing search procedure and keywords, a total of 526 articles were found in the PubMed database, 84 articles in Science Direct database, and 105 articles in the Scopus database, totaling 715 articles. Of the articles that were identified, 64 were duplicates, 473 were not considered further for inclusion based on abstract screening and 166 articles (Appendix 1) were excluded for not reporting the main outcome of the procedure, as detailed in Figure 1. According to defined inclusion criteria, 12 articles were selected for this review.

### **Sources of collagen**

Collagen can be extracted from various sources such as land animals, marine animals, and microorganisms. Widespread applications of collagen in cosmetic, biomedical, and pharmaceutical industries make collagen from skin and bones of bovine and porcine collagen as the major industrial sources of collagen (Cliché *et al.*, 1998).

Based on this systematic review 12 articles were selected, of which 5 were carried out using collagen derived from the recombinant system, 4 using collagen from mammals and 3 described isolation and purification of marine-derived collagen. Two out of five studies reported about collagen-like molecules in bacteria and the rest studies reported about recombinant collagen from animals and non-animal sources.

Recently, instead of focusing on animal-derived collagen, most of the studies focused on

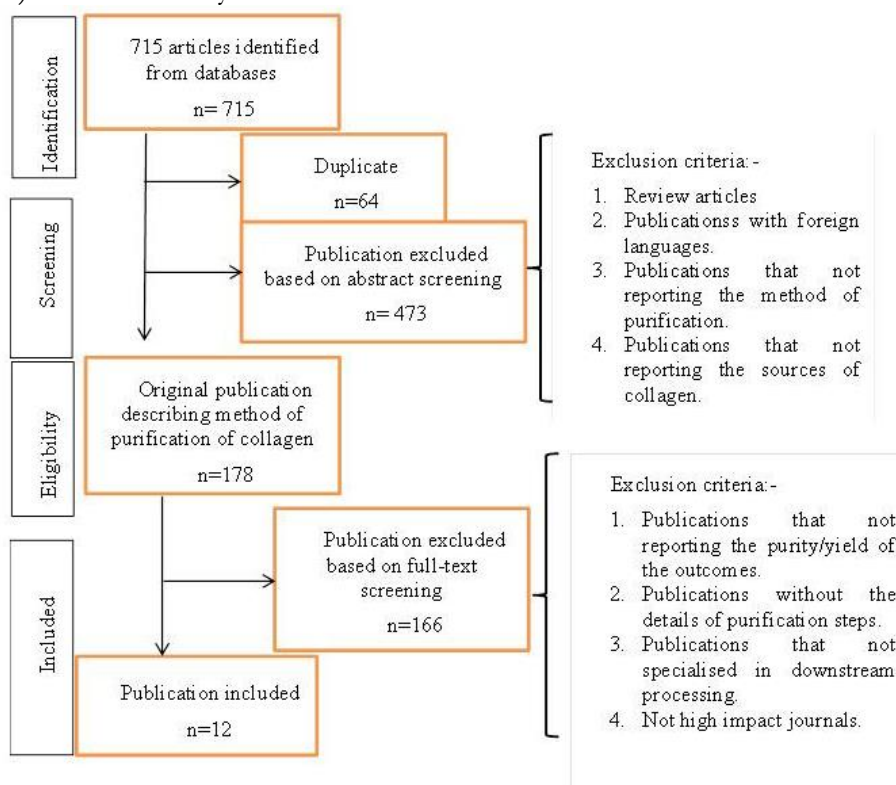
non-mammalian sources such as bacteria and marine sources. Less than half of the publications reported about the purification of collagen from mammalian sources. This is due to the outbreak of transmissible diseases like bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathies (TSE) and foot-and-mouth disease (FMD) that pose threat to human (Jongjareonrak *et al.*, 2005; Peng *et al.*, 2012a; Silvipriya *et al.*, 2015; Singh *et al.*, 2011; Swatschek *et al.*, 2002; Zhang *et al.*, 2009). Furthermore, religious sentiments for Muslims, Jewish, and Hindus created another issue in the consumption of porcine or bovine-derived collagen (Sadowska *et al.*, 2003; Singh *et al.*, 2011; Souza, 2016; Wang *et al.*, 2017). Islamic and Jewish believers are prohibited in the consumption of pork, whereas bovine sources are prohibited for Sikhs and Hindus (Eriksson *et al.*, 2013; Herbert *et al.*, 1994; Singh *et al.*, 2011; Souza, 2016). Considering the potential shortcomings and religious sentiments related to mammalian collagen and marine collagen can be seen as a promising alternative to mammalian collagen since they are free from zoonosis, less immunogenic, high content of collagen, and environmental friendly (Silva *et al.*, 2014; Silvipriya *et al.*, 2015) (Felician *et al.*, 2018) (Applications *et al.*, 2019). Marine based collagen can be extracted from marine sponges (Swatschek *et al.*, 2002), jellyfish (Krishnan & Perumal, 2013), squid (Raman & Mathew, 2014), and fish (Kiew & Don, 2013) and their offal including processing waste such as fish skin, scales, bones, fins, and heads (Maya Raman & Gopakumar, 2018; Sadowska *et al.*, 2003). Thus, these under-utilized sources can be considered as sustainable and cost-effective (Maya Raman & K Gopakumar, 2018; Senaratne *et al.*, 2006).

Nowadays, collagen-like molecules from bacteria have become the attention of researchers and scientists to find another alternative of animal-derived collagen (An *et al.*, 2014; Peng *et al.*, 2012a; Xu *et al.*, 2010, 2014). Collagen proteins from *Methylobacterium* sp. 4-46, *Rhodospseudomonas palustris*, and *Solibacter usitatus* were identified as soluble proteins whereas collagen from *Clostridium perfringens* was found as inclusion bodies (Xu *et al.*, 2010). Interestingly, bacterial collagen can form a triple helix structure through electrostatic interactions and functioned as animal collagen even though they are lacking of post-

translationally modified amino acid, hydroxyproline (Hyp) ) (Peng *et al.*, 2012b). With the absence of hyp, they were stable around mammalian body temperature, with melting temperatures of 36.4°C and 37.6°C (Ramshaw *et al.*, 2014; Xu *et al.*, 2002). Hydroxyproline is responsible in stabilize the triple helix structure of collagen (Berisio *et al.*, 2002). If the stability of

collagen is low, collagen will be denatured and stay in single-chain without folding into the native triple-helical structure. This single structure is known as gelatin (Ramshaw *et al.*, 2014). Out of these 12 articles, only 2 describing bacterial collagen, clearly indicating a need for further research related to bacterial collagen.

**Figure 2.** Flow diagram of the inclusion process.



### **Purification of collagen**

There are many techniques on the purification of collagen has been invented for the past years. The conventional purification methods for collagen are precipitation, filtration, and chromatography (Mazzola *et al.*, 2008).

### **Affinity chromatography**

It has been stated that over 60% of all purification techniques involve affinity chromatography (Magdeldin & Moser, 1996; Przybycien *et al.*, 2004). The principle behind this technique is based on the reversible interactions between the desired protein and affinity ligand coupled to the chromatographic matrix (Magdeldin & Moser, 1996; Pontis, 2017; Urh *et al.*, 2009). During this process, the specific protein will bind to the ligand while non-bound components leave the column before the elution process occurred by altering the buffer conditions (Coskun, 2016). Next, the

bounded molecule will be eluted during the desorption step by altering conditions leading to the binding such as pH or salt concentration (Fujita-Yamaguchi, 2015). The target protein will be collected in a concentrated form.

Purification of collagen using affinity chromatography was reported in 3 out of 12 studies (25%) (Rutschmann *et al.*, 2013; Warner *et al.*, 2007; Xu *et al.*, 2010). These studies involved the purification of different types of collagen from recombinant system technology. There are several types of columns available for the application of affinity chromatography such as zinc, cobalt, copper, and nickel (Magdeldin & Moser, 1996). Those studies used the nickel affinity column for their purification process. One publication reported the procedure of affinity chromatography using a fast protein liquid chromatography (FPLC) system, and sepharose as the chromatographic matrix (Rutschmann *et al.*,

2013). Sepharose is a registered tradename for a cross-linked agarose-based, beaded-form gel matrix (Zucca *et al.*, 2016). FPLC previously known as fast performance liquid chromatography is a medium pressure chromatography that mainly used for purification of proteins and biomolecules. Its stationary phase is composed of small diameter beads which make it different from HPLC (Pontis, 2017). Compared to high-performance liquid chromatography (HPLC), the cost per test for FPLC is 30 times cheaper whereas its column is 10 times cheaper than HPLC column (Gireesh, 2004; Tangvarasittichai *et al.*, 2009). However, the yield of collagen produced using this method is very low which is about 0.009%.

According to Xu *et al.* (2010) and Warner *et al.* (2007), agarose was chosen as the chromatographic matrix for their studies due to its ability to form a highly porous matrix with minimal non-specific adsorption. Agarose is a polysaccharide that originated from seaweeds that able to form spontaneous gelation (Aschenbrenner *et al.*, 2013; Royhaila *et al.*, 2015; Zucca *et al.*, 2016). His-tagged proteins were purified by affinity chromatography Ni-NTA agarose superflow column (Warner *et al.*, 2007; Xu *et al.*, 2010). The expression of histidine-tag recombinant protein allows it to be selectively bound by divalent metal ions (Bolanos-garcia & Davies, 2006; Hochuli *et al.*, 1987; Smith *et al.*, 1988). Then, a high concentration of imidazole was used in the desorption step as an elution buffer to elute the target protein (Fujita-Yamaguchi, 2015; Rutschmann *et al.*, 2013; Warner *et al.*, 2007; Xu *et al.*, 2010). The recovery of collagen is about 11% to 12% (Xu *et al.*, 2010) and 15% (Warner *et al.*, 2007).

Affinity chromatography is most preferable due to its high resolution and high selectivity (Bolanos-garcia & Davies, 2006; Pontis, 2017). All the publications reported the affinity chromatography technique used is immobilized metal affinity chromatography (IMAC) technique and nickel as the immobilized metal ion on the matrix because it gives high yield compared to other types of column (Bolanos-garcia & Davies, 2006; Bornhorst & Falke, 2000). This technique is widely used especially for the single-step purification of recombinant proteins (Block *et al.*, 2009). Affinity-tagged protein purities can be

achieved in high yield at up to 95% purity by IMAC (Bryntesson *et al.*, 2011). However, proteins from *E. coli* host sometimes naturally binds to the nickel ligand and co-eluted with the recombinant protein during the purification process. This problem usually solved by applying a high concentration of imidazole. Protein that was observed to be co-purified with the His-tagged protein can be divided into four groups (Bolanos-garcia & Davies, 2006):

- I. proteins with natural metal-binding motifs
- II. proteins with histidines clusters on their surfaces
- III. proteins that bind to heterologously express His-tagged proteins
- IV. protein with affinity to agarose-based supports

As we know, tagged protein has a higher binding affinity than other proteins that bind non-specifically to the resin. The filtered binding buffer used contained a low concentration of imidazole. Imidazole is a chemical that is very specific to nickel. When the minimum amount of resin used, the tagged protein will fill most of the available binding sites and will decrease the non-specific binding. Conversely, a high concentration of imidazole was used in the elution buffer. The main function of imidazole is to reduce the non-specific binding of a protein to the column. It will compete with the polyhistidine-tagged to the recombinant protein for binding to the column and the purified protein will be eluted out from the column.

Even though affinity chromatography is the most promising method especially for recombinant collagen, there are still serious issues. For example, Block and co-workers reported that the complexity of its procedures and the high cost of maintenance due to the multiple stages involved are viewed as significant disadvantages (Coskun, 2016). Besides, how the affinity tags used may alter the protein activity is a growing concern (Bornhorst *et al.*, 2000; Dimitrova *et al.*, 2017). Proteins with a low level of expressions will not able to incorporate with this method and they require other affinity tags of polyhistidine tag with additional purification techniques (Bornhorst *et al.*, 2000). It is undeniable that this method comes first in the purification of recombinant proteins,

however, it is lacking in terms of productivity and economical value and thus not sustainable for large scale and long-term production.

### ***Ion exchange chromatography***

Ion-exchange chromatography works based on electrostatic interactions between charged protein groups, and solid support material (matrix). There are two types of resins which are cation and anion exchange. Negatively charged proteins will bind to positively charged ion-exchanged matrices and vice versa (Dimitrova *et al.*, 2017). According to Sato and co-workers, filter paper-based Diethyl aminoethyl cellulose (FBP DEAE) column chromatography was used to purify type V collagen from a pepsin digest of porcine intestinal connective tissue (Sato *et al.*, 2003).

One out of twelve (8.3%) of the selected articles reported using ion-exchange chromatography as the method of collagen purification (Sato *et al.*, 2003). Filter paper-based Diethyl aminoethyl cellulose (FBP DEAD) column chromatography is an example of ion-exchange chromatography used to purify type V collagen from the porcine intestine (Sato *et al.*, 2003).

Examples of ion exchanger matrix used in this technique are dextran, polystyrene, cellulose, and acrylic (Coskun, 2016). The introduction of FBP DEAD-cellulose by Sato and co-workers allowed the purification of type V collagen using a rapid and simple way with high yield in a shorter time (Sato *et al.*, 2003). Besides the charge, the pH of the binding buffer also plays an important role in this technique. For a successful binding, the pH value must be below the isoelectric point (pI) of the protein (Dimitrova *et al.*, 2017). According to Henderson-Hasselbalch principle, if the pH of the binding buffer is merely one pH unit below the isoelectric point (pI) of the recombinant proteins, most of the latter will bind to the column (Radić & Prkic, 2012). In the publication reported by Sato and co-workers, 50 mM Tris–HCl buffer at pH 8.20 was used which is below the isoelectric point (pI) of collagen, 8.26. Instead of improving the specific binding, further decreasing the pH of the buffer will invite the contaminating proteins to bind (Dimitrova *et al.*, 2017).

According to Sato and co-workers, this technique is able to separate the proteins with high concentration without involving clogging

problems. Furthermore, utilization of FBP DEAE-cellulose can be considered as a cost-effective method as it is reusable and can be prepared inexpensively using available glassware and reagent such as sodium hydroxide, hydrogen chloride, sodium chloride and 2-chlorotriethylamine (Sato *et al.*, 2003). However, the preparation of the experiment is tedious and high possibility of contamination to occur. Examples of contamination that commonly occurred are organic and bacterial contamination from the resin (sciencing) (Block *et al.*, 2009). Same goes to affinity chromatography, along with its ability to produce high purity of the product, ion exchange chromatography is a costly and tedious technique that require expensive chemicals and equipment. Hence, a simple yet efficient technique should be a better choice.

### ***Precipitation***

Precipitation is one of the conventional methods of purification that is widely used in the downstream processing of biomolecules. Precipitation techniques that commonly used in collagen purification are salt precipitation and acid precipitation. Despite its tedious procedures, this method is still applicable in the industry as it is inexpensive compared to other methods.

Three out of 12 (25%) studies used precipitation technique in their procedure. Two out of three publications reported on acid precipitation (Singh *et al.*, 2011; Werkmeister & Ramshaw, 2015) and the rest conducted the collagen purification using salt precipitation (Cao & Xu, 2008). These methods required additional purification processes such as chromatography and pepsin digestion.

Purification of recombinant bacterial collagen using a combination of precipitation and proteolysis by pepsin was performed in two out of three studies (Cao & Xu, 2008; Werkmeister & Ramshaw, 2015). Werkmeister, Singh, and co-workers reported that the application of precipitation and proteolysis is applicable to a wide range of bacterial collagen with different collagen domain sizes (Singh *et al.*, 2011; Wang *et al.*, 2017). The acid precipitation method can be considered as an efficient technique in removing a high amount of bacterial host proteins and they will tend to precipitate upon the addition of acid as host proteins were least soluble at a pH lower

than 5 (Kiew & Don, 2013; Werkmeister & Ramshaw, 2015). However, the significant drawback is the acidic conditions itself may lead to adverse effects on the stability of the triple helix in these bacterial proteins (Werkmeister & Ramshaw, 2015).

The formation of contaminants alongside the salting-out process is the major challenge in salt precipitation. According to Cao & Xu, a combination of pepsin digestion, salt precipitation by NaCl, and ion-exchange chromatography were used to purify type II collagen from chick sternal cartilage (Cao & Xu, 2008). Thus, additional purification process such as chromatography is needed to obtain a high purity protein. Moreover, the involvement of pepsin in the precipitation process will indirectly lead to a high cost of operation due to the high cost of enzymes. Even though precipitation is an easy and inexpensive method, it did not gain in popularity due to its main weakness in producing low purity of proteins. An improvement regarding this method is needed to compete with other purification methods.

### **Aqueous two-phase system**

Aqueous two-phase system (ATPS) or aqueous biphasic system (ABS) is a liquid-liquid fractionation technique and has been used for many years in biotechnological applications specifically for separation and purification of biological materials such as proteins, enzymes, nucleic acids, virus, antibodies and cell organelles (Iqbal *et al.*, 2016; *et al.*, 2011). ATPS is formed when two aqueous phase consists of two water-soluble polymers or a polymer and a salt that are mixed at appropriate concentrations (Grilo *et al.*, 2014; Raja *et al.*, 2012; Rito-palomares *et al.*, 2014; Song *et al.*, 2013). Then, the mixture will result in two liquid layers with a high content of water (Tjerneld, 1994). In 2002, a new ATPS system has been applied in the purification of glycyrrhizin from *Glycyrrhiza uralensis* Fisch using alcohol and salt instead of polymer and salt system (Tianwei *et al.*, 2002).

Prior to this purification process, a unique diagram known as a phase diagram under a specific condition of pH, temperature, or salt concentration needs to be constructed. It is a crucial diagram that provides the information about the concentration of phase-forming

components and consists of a binodal curve that separates the region with the biphasic solution (above the curve) from the one that does not (below the curve) (Hatti-Kaul, 2000; Iqbal *et al.*, 2016; Johansson *et al.*, 2011; Raja *et al.*, 2012; Silva *et al.*, 2014; Stitt & Weatherley, 1994; Tjerneld, 1994). Protein partition in ATPS is a complex phenomenon due to the involvement of many factors including the molecular weight of polymers, and electrochemical and ionic properties of the phases (Iqbal *et al.*, 2016; Jankowski, 2012; Mazzola *et al.*, 2008; Ratanapongleka, 2013; Creighton *et al.*, 1989). The main goal is to localize the target protein in one phase either top or bottom phase and the partition coefficient at each phase will be used to evaluate the purification efficiency (Singh & Tavana, 2018).

Purification of collagen using the aqueous two-phase system was reported in 1 out of 12 (8.3%) studies. The system composed of non-ionic polymers polyethylene glycol (PEG) and dextran (DEX) to purify type I rat tail collagen. Table 1 shows 4 types of ATPS system (A, B, C, D) that has been tested in this study. PEG and DEX are the most commonly used in ATPSs among polymers since they can perform desirable physical properties with non-toxicity and applicable for microbial cells due to its stabilizing effect (Ratanapongleka, 2013). Collagen partition is highly sensitive to the molecular weight of polymer which is the factor that can affect the partition of biomolecules (Singh & Tavana, 2018).

**Table 1.** Aqueous two-phase system used in collagen partition (Singh & Tavana, 2018).

System	Polyethylene Glycol (PEG)	Dextran (DEX)
A	PEG8000	DEX40000
B	PEG8000	DEX500000
C	PEG35000	DEX40000
D	PEG35000	DEX500000



**Table 2.** Characteristics of included publications regarding purification of collagen.

Reference	Sources of collagen	Method	Advantages	Disadvantages	Yield
(Sato <i>et al.</i> , 2003)	Porcine intestine (type V collagen)	Filter-based paper DEAD –cellulose column chromatography (ion-exchange chromatography)	<ul style="list-style-type: none"> <li>• inexpensive</li> </ul>	<ul style="list-style-type: none"> <li>• column chromatography need to be prepared manually</li> </ul>	Qualitative
(Werkmeister & Ramshaw, 2015)	Recombinant non-animals collagen	<ol style="list-style-type: none"> <li>1. Acid precipitation</li> <li>2. Proteolysis (pepsin)</li> </ol>	<ul style="list-style-type: none"> <li>• Simple and effective</li> <li>• Inexpensive</li> <li>• Easy to scale up</li> </ul>	<ul style="list-style-type: none"> <li>• Acidic condition can affect the stability of collagen-triple helix structure</li> <li>• Further polishing steps needed for biomedical application</li> </ul>	Qualitative
(Rutschmann <i>et al.</i> , 2013)	Human collagen	Affinity chromatography	<ul style="list-style-type: none"> <li>• High selectivity</li> <li>• High purity</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive</li> <li>• Low yield</li> </ul>	Quantitative & Qualitative Yield=0.009%
(Warner <i>et al.</i> , 2007)	Recombinant	Nickel affinity chromatography	<ul style="list-style-type: none"> <li>• High selectivity</li> <li>• Reduce the risk of cross-contamination (prepacked column)</li> </ul>	<ul style="list-style-type: none"> <li>• Low recovery</li> <li>• Expensive</li> <li>• Complex to sale up</li> </ul>	Quantitative Yield = 15%
(Glatz, 2009)	Corn grain-derived recombinant collagen	<ol style="list-style-type: none"> <li>1. Membrane ultrafiltration</li> <li>2. Chromatography (4 steps) (IEC)</li> </ol>	<ul style="list-style-type: none"> <li>• High yield and purity</li> </ul>	<ul style="list-style-type: none"> <li>• Pore blockage problem</li> <li>• Complex process</li> </ul>	Quantitative Yield = 16 %
(Singh & Tavana, 2018)	Rat tail collagen : Type I	Aqueous two-phase system	<ul style="list-style-type: none"> <li>• Simple and benign</li> <li>• Cost-effective</li> <li>• Rapid separation</li> </ul>	<ul style="list-style-type: none"> <li>• Not easy to scale up because PEG can cause corrosion of equipment</li> </ul>	Quantitative Sys A: 62% Sys B: 34% Sys C & D :58%



(Xu <i>et al.</i> , 2010)	Recombinant Bacteria	Affinity chromatography	<ul style="list-style-type: none"> <li>• High selectivity</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive</li> <li>• Low recovery</li> </ul>	Quantitative & Qualitative Yield: 11-12 %
(Yd <i>et al.</i> , 2013)	Sea cucumber ( <i>Bohadschia bivitatta</i> )	Pepsin-solubilized collagen (PSC)	<ul style="list-style-type: none"> <li>• High yield compared to acid precipitation</li> </ul>	<ul style="list-style-type: none"> <li>• Religious sentiments</li> </ul>	Quantitative Yield= 65%
(Jiang <i>et al.</i> , 2009)	Jellyfish	Ultrafiltration	<ul style="list-style-type: none"> <li>• Eco- friendly</li> <li>• Energy saving</li> <li>• Low maintenance</li> </ul>	<ul style="list-style-type: none"> <li>• Low yield/recovery</li> </ul>	Quantitative Yield= 4.2%
(Cao & Xu, 2008)	Cartilage of chick	<ul style="list-style-type: none"> <li>- pepsin digestion</li> <li>- NaCl precipitation</li> <li>- Ion exchange chromatography</li> </ul>	<ul style="list-style-type: none"> <li>• High purity</li> </ul>	<ul style="list-style-type: none"> <li>• Time-consuming</li> </ul>	Qualitative
(Singh <i>et al.</i> , 2011)	skin of striped catfish ( <i>Pangasianodon hypophthalmus</i> )	<ul style="list-style-type: none"> <li>- Acid Precipitation</li> <li>- Porcine pepsin</li> </ul>	<ul style="list-style-type: none"> <li>• No effect on triple helix structure</li> </ul>	<ul style="list-style-type: none"> <li>• Collagen not completely solubilized in acid</li> </ul>	Qualitative and Quantitative ASC: 5.1% PSC: 7.7%

DEAD, diethylamino ethyl: IEC, ion-exchange chromatography: ASC, acid-solubilized collagen: PSC, pepsin-solubilized collagen: PEG, polyethylene glycol.

Although PEG and DEX system are commonly used, the high cost of fractionated dextran is a limitation for its application in a large scale process (Ratanapongleka, 2013). Another system that composed of one polymer and low molecular weight of salt is usually preferred especially in large scale operation due to the low cost of salt compared to dextran (Ratanapongleka, 2013; Rito-Palomares, 2004). Furthermore, the polymer-salt system provides larger differences in density, lower viscosity, and greater selectivity (Ratanapongleka, 2013; Song *et al.*, 2013). However, in terms of the economical aspect, the salt/alcohol system comes first due to the easy recovery of alcohol using the evaporation method (Tianwei *et al.*, 2002). According to data from Table 2, the yield of collagen extracted using this technique is quite high which is 62% for system A, 34% for system B, and 58% for system C and D (Singh & Tavana, 2018). There are many advantages of aqueous-two phase system which are simple, benign, easy to scale up with rapid mass transfer and excellent separation with little denaturation that make it as a promising substitute for the existing method which is expensive and classical, such as chromatography (Asenjo & Andrews, 2012; Azevedo *et al.*, 2007; Sarubbo *et al.*, 2004; Silva *et al.*, 2014; Song *et al.*, 2013). Furthermore, they are preferable in biomedical research studies due to its biocompatibility and eco-friendly because they are free from any organic components (Brunette & Till, 1971; Ling *et al.*, 2010; Song *et al.*, 2013).

The phase separation process only needs one step which is the centrifugation step. A direct comparison between ion-exchange chromatography and aqueous two-phase techniques has been conducted by Rito-Palomares, proved that ATPS method able to reduce the number of unit operation (from 7 to 4) thus leads to further reduction of the cost by 43% (Rito-Palomares, 2004).

However, the main disadvantage of this method is the lack of knowledge on the mechanism involved in the partitioning process and poor understanding of the technique (Rito-Palomares, 2004). Besides, environmental issues such as salt disposal and their impact on wastewater treatment remain the problem on large scale ATPS process (Ratanapongleka, 2013). Although simple and economical, ATPS has yet

to see widespread of collagen purification. In my opinion, if the mechanisms of protein partitioning are deeply studied and understand, ATPS can be a good alternative for large scale protein purification especially collagen.

### **Ultrafiltration**

The basic understanding of this method is the removal of contaminants by passing the sample through a permeable medium but traps the contaminants or solid particles (Shon *et al.*, 2011). This method of purification is widely used in a water treatment plant in the environmental industry. It is commonly used as a pre-concentration step in the purification of protein solutions (Machado *et al.*, 2018; van Reis & Zydney, 2007). Several research studies have applied this approach include fractionation of whey protein (Cheang & Zydney, 2004), purification of DNA (Kahn *et al.*, 2000), and lysozyme (Ghosh & Gui, 2000). However, few studies reporting about collagen purification using the ultrafiltration method (Cao & Xu, 2008; Jiang *et al.*, 2009).

Purification of collagen using the filtration method has been outlined in 2 out of 12 (16.7%) publications. A membrane made up of polyacrylonitrile (PAN) and hydrophilically modified polyacrylonitrile was used to purify the collagen from jellyfish (Jiang *et al.*, 2009). The utilization of hydrophobic material alone like polyacrylonitrile is problematic due to the rapid fouling during the separation process (Minnesota Rural Water Association, 2001), (Ramli *et al.*, 2014). Hence, hydrophilic and charged membranes are the solutions discovered in recent years to avoid membrane fouling.

Glatz (2009), outlined the purification of corn-grain derived recombinant collagen using a combination of membrane ultrafiltration, non-affinity chromatography, and low pH extraction. Polyethersulfone membranes with 30, 50, and 100 kDa molecular weight cut off (MWCO) were used in the procedures to identify the most potent membrane (Glatz, 2009). The 50kDa membrane was found to be the most effective membrane with higher flux and shorter processing time that contributed to about 80% removal of contaminating proteins (Glatz, 2009).

Ultrafiltration is known as an energy-saving and eco-friendly technique that able to produce

high product throughput compared to other conventional separation methods (Jiang *et al.*, 2009). However, the utmost difficulty in ultrafiltration is membrane fouling that will affect membrane performance and leads to the high cost of maintenance (Aimar & Bacchin, 2010; Ramli *et al.*, 2014; Tang *et al.*, 2011). Mechanisms of membrane fouling consist of physical and chemical adsorption, precipitation of salts, and the deposition of biofilms and suspended matter onto or into the membrane (Akhondi *et al.*, 2017). Other than that, cross-contamination is a most likely problem occurred in concentration equipment such as stirred ultrafiltration cells with ultrafiltration membranes (Lingala & Ghany, 2016). Furthermore, this method is not suitable for the separation of low molecular weight species (Tang *et al.*, 2011). Despite these circumstances, ultrafiltration still possess great potential in the purification process especially in the water treatment industry and biotechnology industries such as in protein fractionation (Machado *et al.*, 2018).

About 4.2% of collagen produced by a study carried out by Jiang and co-workers, which is relatively low (Jiang *et al.*, 2009). Meanwhile 16% of collagen able to be purified using a combination of ultrafiltration with non-affinity chromatography and low pH extraction method. Therefore, an additional purification step is needed in this case to obtain a pure and high yield of proteins. For collagen purification, ultrafiltration is not a suitable choice, possibly because of its multi-stages operation to purify the protein from a complex mixture.

## CONCLUSION

In conclusion, many different techniques have been discovered for the purification of collagen. The choice of the purification method mainly relied on the sources, types, and structure of the collagen itself. For instance, affinity chromatography commonly used for purification of recombinant collagen as it has been genetically modified and appended by affinity tags. Meanwhile, the conventional purification method is preferable for non-recombinant collagen from mammalian and marine sources. Notably, out of

12 publications reporting collagen purification, purities and yield were not quantitatively measured in 3 publications. Reporting the yield is very important that will lead to a meaningful interpretation of the results. Furthermore, the cost of analysis of each method used was not reported in any publication. From economic point of view, adding cost analysis could be a good improvement in publishing journals regarding the purification technique.

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