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Determination of the bands of four common animal collagens by SDS-PAGE electrophoresis and the comparative study of their protein functional regions

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Keywords: Electrophoresis; collagen; SMART; evolution.

Abstract: Collagen is the most abundant protein in animals, which is widely distributed in all kinds of animals, and its composition and evolution is very complex. In this study, SDS-PAGE protein electrophoresis was used to determine the changes of electrophoretic bands of collagen extracted by enzymatic hydrolysis from pigs, cattle, fish and rats, and the results of electrophoretic hydrolysis are summarized below. Hydrolysis from pigs, cattle, fish and rats, and the results of electrophoretic bands were classified and sorted out. Using the different characteristics and proportion data of collagen electrophoretic bands of four kinds of animals, the characteristic bands were analyzed, and the Using the different characteristics and proportion data of collagen electrophoretic bands of four kinds of animals, the characteristic bands were analyzed, and the functional region analysis and prediction tool of SMART protein was used. The relevant research results provide a reference for clarifying the characteristics of collagen evolved in different animals and for the establishment of its determination methodology, and for the determination of collagen in different animals. The relevant research results provide a reference for clarifying the characteristics of collagen evolved in different animals and for the establishment of its determination methodology, and provide experimental data and scientific basis for the development of different types of collagen medical products.

1. Introduction

Collagen is an animal connective tissue. The collagen content accounts for about 20% of the protein in mammals and is also the main component of Collagen. As a kind of biomolecule polymerized protein, it is the most abundant and widely distributed functional protein in the body of mammals. Collagen is also a complex family of proteins rather than a single protein[1]. More than 16 types of collagen molecules can be formed in the 30 genes coding for collagen. With so many collagen types containing large and small collagen subunits, collagen molecules play an extremely important role in life functions.

There are many types of collagen. If categorized according to their structure, the common types are type I, type II, type III, type V and type XI[2]. If classified according to its function, the types of collagen can be divided into fibrillar collagen, basement membrane collagen, anchoring collagen,

hexagonal mesh collagen, non-fibrillar collagen, transmembrane collagen and so on. Collagens are also widely distributed, both inside and outside the cell. According to their distribution and composition in the body and functional characteristics, collagen can also be divided into interstitial collagen, basement membrane collagen and peripheral cell collagen. Interstitial collagen molecules account for most of the collagen in the entire body, including type I, II and III collagen molecules, most of which belong to interstitial collagen molecules. Type I collagen, whose thermal stability is not high, is mainly distributed in the skin, tendons and other exterior tissues and root tendon connecting tissues, and it is one of the most abundant collagen in both the animal body and the processing waste of animal products, accounting for about eighty to ninety percent of the content of all collagen. It is also widely used in medicine. Type II collagen is produced by chondrocytes and belongs to the interstitial collagen tissue. Type IV collagen mainly constitutes a component of the collagen basement membrane. Extracellular peripheral collagen is widely distributed outside the cell and is mainly composed of type V collagen. This extracellular peripheral collagen is widely distributed in connective tissue.

Collagen consists of a typical triple helix structure, and these collagen triple helices are often attracted to each other through hydrogen bonds or through interchain disulfide bonds to build the complex spatial structure of collagen[3]. Collagen molecules are highly extensible and elastic, generally in the form of elongated rods, with relative molecular masses, ranging from about 2kD to 300kD. Collagen has good water retention, emulsification and film-forming properties. Therefore, it can be used as raw materials for cosmetics[4].

Collagen is susceptible to hydrolysis by animal collagenases, which are widely distributed in the environment, resulting in artificially extracted collagen that is often prone to breakage of large segments. Collagen itself is one of the most complex classes of proteins. Due to its properties of being highly susceptible to enzymatic hydrolysis and undergoing fracture, collagen also has one of the most complex electrophoretic profiles. It is generally recognized that collagen has a molecular weight of about 300KD, and electrophoresis will show bands at 100KD or 200KD, respectively. That is, most α chains are at 100KD, β chains at 200KD, and γ chains at 250KD[5].

Collagen is widely available and inexpensive, biocompatible and biodegradable, and has very low immunogenicity. Therefore, collagen has a wide range of prospects for application in materials used for tissue damage repair, cosmetic make-up masks and other materials. Collagen is an important source of raw materials for various cosmetic and biomedical products. Every year, a large amount of animal carcass trimmings are disposed of in farms and slaughterhouses. If these trimmings are not handled carefully, it is easy to cause pollution and spread disease. Therefore, the use of these animal carcass trimmings to develop a variety of active bio-collagen products can not only reduce the burden on the environment, but also has a very high medical value. In recent years, more and more collagen products have been promoted and applied to the health and beauty markets, showing a promising future in the regeneration and repair of diseased and defective tissues and organs, as well as in the beauty and make-up industry. However, collagen is a purely natural compound from different animal sources and ages, and the quality of collagen obtained from different extraction methods and processes varies, resulting in the development of the same health and beauty products with different effects[6]. Therefore, it is necessary to understand the evolution of collagen from different animal sources to provide a reference for the selection of reasonable extraction methods and a scientific basis for the development of health and beauty products with more uniform quality.

In this study, the SDS-PAGE electrophoresis method was selected, combined with silver staining and coomassie brilliant blue staining. By determining the changes in the electrophoretic spectral bands of porcine tendon proteins, bovine tendon collagens, fish skin collagens, and rat tail collagens enzymatically extracted from four kinds of animals: pig, cow, fish, and rat, and the data of the

results of electrophoretic bands were categorized and organized, and the data on the electrophoretic bands of the collagens from the four animals obtained were utilized for the characterization of the electrophoretic bands and the proportion data The data were categorized, and the data on the characteristics and proportions of the collagen electrophoretic bands obtained from the four animals were used to study the correlation of collagen evolution in the four different animals. The results of this study are useful for clarifying the characteristics of collagen evolution in different animals and providing reference for the establishment of methodology for its determination, as well as providing experimental data and scientific basis for the research and development of different types of collagen-based medical products based on the four animal species.

2. Methodology

2.1 Collagen extraction

2.1.1 Porcine Achilles tendon collagen extraction

Pork Achilles tendon pellets were washed and crushed into powder. Then the pork Achilles tendon particles were degreased with a degreasing solution and then put into a concentration of 0.1-0.5% acetic acid with pepsin. Put into the ratio of every 100-150 mg of 0.1-0.5% acetic acid solution add 5-10 grams of pig Achilles tendon particles and 1-5 grams of pepsin and mix them well, and then magnetic stirring for 30-60 hours to get the enzymatic solution. The obtained enzyme solution was filtered with 100-mesh gauze to remove dregs, and then vacuum filtered. The filtered enzyme solution was centrifuged with a centrifuge, and the supernatant collagen solution crude was taken. The crude collagen solution obtained was first dialyzed with 0.050-0.155 M acetic acid solution for 40-50 hours, and then dialyzed with distilled water for 20-25 hours, and the final remaining dialysate was the collagen extract[7-9].

2.1.2 Fish skin collagen extraction

Grass carp skin to remove phosphorus, cut and wash, in 10-20 times the raw material weight containing a volume fraction of 0.5-3% H O_{22} NaOH solution stirring immersion 24-48h, wash to neutral; continue to add isopropanol solution stirring for 4h, and then wash with purified water for 3 times, and then add sodium chloride solution stirring for 12h, centrifugation, to remove the waste liquid that is the pretreatment of hydrolyzed crushed fish skin. Pretreatment of crushed fish skin and deionized water to the mass ratio of 1:5-1:10 mixing, homogenization, adding pepsin, hydrochloric acid pH = 2.5, enzyme digestion 20h, filtration, 8000-20000rpm centrifugation for 20 min, in the supernatant add sodium chloride to the final salt concentration of 0.9 mol/L, salting out, 8000 rpm centrifugation for 20 min, discard the supernatant, and then obtain the enzymatic hydrolysis of collagen. The enzymatically hydrolyzed collagen was obtained. Subsequently, the precipitate was dissolved by adding 10 times the volume of 0.5 mol/L acetic acid solution, filtered to remove insoluble impurities; then dialyzed in 10 times the volume of 0.1 mol/L acetic acid solution for 12-24 h, and the solution was changed every 3 h. Finally, it was dialyzed with purified water to neutrality, freeze-dried, crosslinked, and purified water immersed and cleaned for 5-10 times, and then freeze-dried to obtain the collagen from fish skin[10-11].

2.1.3 Beef tendon collagen extraction

Fresh beef tendon washed with ultrapure water, crushed and sliced. Soak in degreasing solution for degreasing, mechanical agitation, using 100 mesh sand net filter, remove and wash again with ultrapure water. Soak the treated tendon in alkaline solution to remove impurity protein, and then

mechanically stir, replace the alkaline solution every 6-8 hours, filter the filtrate through stainless steel mesh to remove the filtrate, and then wash with ultrapure water until the surface of the tendon pH 7. Stir the tendon after removal of impurity protein, and then put it into 1L of acid solution to homogenize into minced meat. Put the powder into a stainless steel container, continue to add 1L of acidic solution, and then add appropriate amount of active pepsin, stirring digestion, centrifugation for 15-30min, and take the supernatant for spare. The acid in the supernatant was neutralized to neutral with NaOH solution, sodium chloride was added and stirred for 15-30min, and the precipitate was the crude collagen type I. Then the precipitate was rinsed with ultrapure water for several times, drained, and then the drained precipitate was dissolved in dialysis solution again, and then dialysis was carried out. After dialysis, the crude liquid is dried and vacuum encapsulated [12-14].

2.1.4 Rat tail collagen extraction

The rat tail was skinned, washed with ultrapure water and pulverized in a pulverizer. Soak with NaOH alkaline solution to remove impurity protein, then mechanical stirring, replace the alkaline solution once every 8 hours, and change it for 3 times in total, remove the filtrate by stainless steel mesh filtration, remove it and wash it with ultrapure water to pH 7. Put it into 1L of acidic solution and homogenize it to become surimi. Put the powder into a stainless steel container, continue to add 1L of acidic solution, then add appropriate amount of active pepsin, stirring digestion, centrifugation at 1000 rpm for 30min, and take the supernatant for spare. Add NaOH solution slowly, neutralize the acid in the supernatant to neutral, add sodium chloride and stir the saline dialysis for 30min, then rinse the precipitate with ultrapure water for several times, and the precipitate is dissolved in the dialysis solution again, and then dialysis is carried out. Rat tail collagen standard, purchased from sigma company, the item number is C7661, the standard is 2.5mg/ml.

2.2 Electrophoretic determination of collagen

(1) Sample preparation:

The samples were dissolved in 3% acetic acid and the proteins were diluted to a concentration of 1 mg/ml for 24h.

(2) Reagent preparation:

Electrode buffer, test buffer (5×), separating gel solution, concentrating gel solution, fixation solution, Chuangmeng silver staining kit ZD303-1.

(3) Measurement:

① BCA quantification for protein concentration.

② Separation gel solution is poured into the mold to a certain height, capped with water, and polymerized at room temperature.

③ After the separation gel is polymerized, the filter paper is sucked to remove the water layer on top, and then the concentrated gel solution is filled in, and the sample comb is inserted, taking care to prevent the appearance of air bubbles.

④ Sample addition: the volume of the upper sample is 10ug. Mix the test material and the test buffer in the ratio of 4:1, and heat it in a boiling water bath for 5 minutes. Carefully pull out the sample comb after the concentrated gel solution has been polymerized, and before and after the electrode buffer has filled the electrophoresis tank, add the test solution in the test material well.

⑤ Electrophoresis: Turn on the power [15].

(4) Fixation and Staining

After electrophoresis, the gel was cut and immersed in a fixative containing 50% ethanol and

10% acetic acid for 30 min, and the fixative was discarded. Protein kojic acid staining was performed according to the following step-by-step procedure:

① Preparation of Caulmers Brilliant Blue dye solution: 0.1% R250 (dissolved in water)

② At the end of electrophoresis, the gel was cut and immersed in a fixative containing 50% ethanol and 10% acetic acid for 30 minutes

③ Soak the gel in 0.1% R250 Khomas Brilliant Blue Stain Solution for 1h

④ Dyeing solution recovery, the gum is immersed in a rinsing solution containing 40% methanol and 10% ethanol, and the rinsing solution is changed every 20 min, generally rinsed 3 times.

Gel imaging was analyzed and photographs were documented for preservation. Gel bands were quantified and analyzed using Image-J[16].

2.3 Bioinformatics analysis and summarization

There are many types of collagen, and the common types are type I, type II, type III, type V, and type XI, so this analysis was based on the most common collagen analysis. Prediction and querying of protein structural domains and functions were performed using the Smart Protein Functional Structure Domain Prediction and Querying Online Tool (http://smart.embl-heidelberg.de/), where sequences were pasted for prediction and all sequence feature prediction options were checked.

Collagen type I α1 chain: grass carp (XP_051743565.1), bovine (NP_001029211.1), porcine (XP_020922812.1), rat (AAI33729.1); α2 chain: grass carp (XP_051729126), bovine (NP_776945.1), porcine (NP_ 001230584.1), rat (XP_032762867.1);

Collagen type II alpha chain: grass carp (XP_051760270), bovine (NP_001001135), porcine (XP_020948270), rat (NP_001401825.1);

Collagen type III alpha 1 chain: grass carp (absent, most aquatic animals lack type III collagen), bovine (NP_001070299.1), porcine (NP_001230226.1), rat (NP_114474.1);

Collagen type V α1 chain: grass carp (XP_051733786.1), bovine (XP_024855494.1), porcine (NP_001014971.1), rat (NP_604447.2); α2 chain: grass carp (XP_051752883), bovine (XP_024835542) porcine (XP_020930354), rat (NP_001386124.1); α3 chain: grass carp (XP_051743414), cattle (NP_001192553), pig (XP_020937095), rat (NP_068528.1);

Collagen XI type α1 chain: grass carp (XP_051738480), bovine (NP_001159981), porcine (XP_001929407), rat (P20909.2) α2 chain: grass carp (XP_051727914), bovine (NP_001039664), porcine (XP_020954165), rat (NP_ 001388231.1).

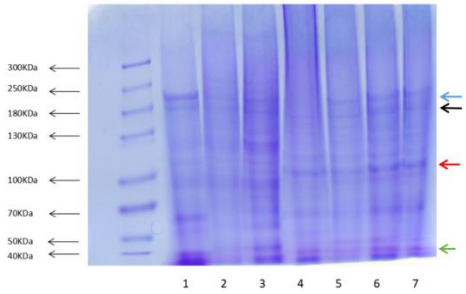
3. Results

3.1 Comparative analysis of collagen bands of four different species

The results of electrophoretic determination of collagen extracted from grass carp, bovine tendon and porcine tendon are shown in Figure 1, and the results of electrophoretic determination of tail collagen extracted from rat and rat collagen commercial standard are shown in Figure 2. The electrophoretic profiles of the extracted collagen from rat and the rat collagen standard were highly similar, which indicated that they were from the same source.

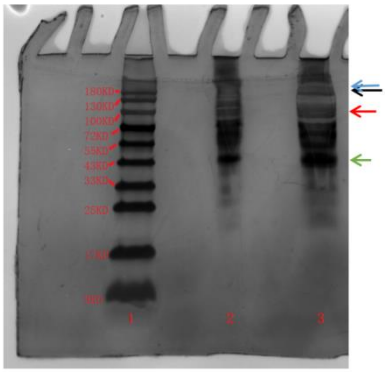
In the range of 180-250 KD, collagen extracted from grass carp, bovine and porcine tendons, and rat tail after enzymatic digestion had the same spectral bands (marked by black arrows), and the clarity of the bands was positively correlated with the purity of collagen, the higher the purity the clearer the bands, and the spectral bands (marked by black arrows) were cross-species, with similar bands of collagen extracted from the four animal species. Therefore, the determination of collagen bands by SDS-PAGE electrophoresis can identify the purity and protein class of collagen extracted from different animal tissues. In the comparison of extraction methods, enzyme extraction (lane 1

versus lanes 2 and 3), although more costly, resulted in clearer bands, meaning that the use of enzymes resulted in purer collagen and less damage to the collagen. Acid extraction, on the other hand, is the most damaging to the proteins, and the excessive use of acid in the acid extraction step is highly susceptible to protein degradation (lane 2, 3 comparison). Especially in the uppermost layer, the most loss was seen in the collagen bands with large molecules of about 180 KD or more. This shows the limitations and destructiveness of acid treatment on collagen macromolecular bands.



1. Enzymatic extraction of collagen in fish skin; 2. Acid extraction of collagen in fish skin; 3. Hydrolysed collagen from fish skin; 4. Enzymatic extraction of collagen from beef tendon; 5. Purified collagen from beef tendon; 6. Enzymatic extraction of collagen in pork tendon; 7. Purified collagen from pork tendon

Figure 1 Caumas Brilliant Blue electropherograms of collagen from grass carp skin, beef tendon, and pork tendon.



1: Protein Marker 2: Mouse Collagen Purified Sample 3: Mouse Collagen Standard

Figure 2 Electrophoresis of rat tail collagen and rat collagen standards

Fish skin collagen differs considerably from pig and bovine collagen in the 70 to 130 KD spectral range. In this 70 KD range (marked by blue arrows), fish skin collagen has extremely distinct bands, while rat, pig and bovine have them, but they are not as distinct as fish. At 100 KD (marked by red arrows), there is a more pronounced marker band in rats, pigs and cattle, but it is less pronounced in fish. And at about 40 KD, the rat has obvious bands, occupying more than 50% of the content of the bands in the whole lane (marked by green arrows). Although similar bands appeared at the corresponding positions in the pig, cow and fish, the content at this position, none of them could reach the high content of the rat, and the same situation appeared in the rat collagen standard (as show in Table 1).

In addition, even the spectral bands between porcine collagen and bovine collagen are not exactly the same, in the 100KD molecular weight red arrow region, with the same treatment, the porcine band seems to be more obvious, basically 2 times the amount of the above band. That is, in the 100KD region, pig collagen has a more distinct characteristic band with a larger amount. This reflects the subtle differences in collagen across species respectively. This subtle difference also reflects the evolutionarily different relationship between collagen mouse, pig and cow. The above

results show that, from the species evolutionary analysis of fish, the collagen of rats and pigs and cattle collagen differences, from the evolutionary analysis of pigs, cattle and rats belong to mammals, closer relatives, but rats also have their own collagen characteristics, and rats, pigs and cattle collagen, the internal differences between the three is a little smaller than fish. Since the differences are so obvious, this also suggests that it is feasible to identify the species origin of each of the above collagen products if it is done by electrophoresis.

Table 1 Collagen, purified collagen from the same treatment steps in rat, pig and bovine (Figure 1 lane 5, lane 7, Figure 2 lane 3), from top to bottom, differences in quantitative analysis of the bands at the blue, black, red and green arrows.

	Relative proportion in cattle	**Relative proportion of pigs**	**rats**
(Fig. 1 lanes 5 and 7, Fig. 2 lane 3. From top to bottom, blue, black, red, and green) Proportion of band volume	0.521	0.531	0.414
	0.505	0.508	0.557
	0.626	1.000	0.731
	0.143	0.182	3.500

3.2 Collagen sequence alignment and bioinformatics analysis

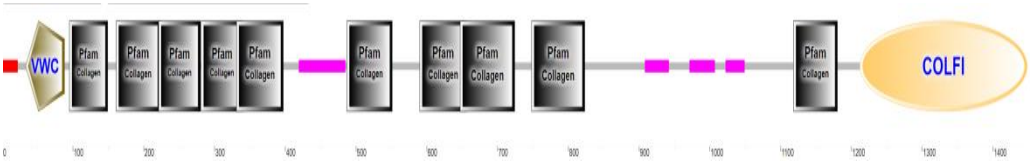
Collagen has multiple types, each of which will have a different molecular weight and electrophoretic profile. In addition, collagen also has multiple types and subunits, and the inconsistent molecular weight sizes of the different types of subunits will likewise affect the pattern during electrophoresis. The collagen profile, therefore, should show image specialties with multiple bands. Based on the results of the electrophoretic mapping, there should be greater differences in the types of collagen from one another where the bands are inconsistent, or where the band profiles are more different. Where the bands are more consistent, there should be a greater likelihood of collagen conserved regions, or conserved subunit sequences. Based on the electrophoretic profile and collagen molecular weight size, the protein sequences of the places where the bands are predicted to be potentially divergent will be compared, and their responses to the collagen differences and species evolutionary relationships, and the results of the comparison of the relationship of the differences are of great significance for the identification of collagen-based protein products.

According to the above SDS electrophoresis results, the fragments with the largest differences are in the range of 70-130 KD bands. Collagens generally have molecular weights between 100-300 KD, with the most common type being around 200 KD. Collagen is mostly linked by disulfide bonds, and on reduced SDS-PAGE, the molecular weight of the single chains formed is also often in the range of 70-100 KD. The 2 bands appearing near 100 KD are the α1 and α2 chains of the collagen molecule, and the 1 band appearing near 200 KD is the β chain of the collagen molecule. Therefore, the results of the present electrophoresis are quite consistent with this conclusion.

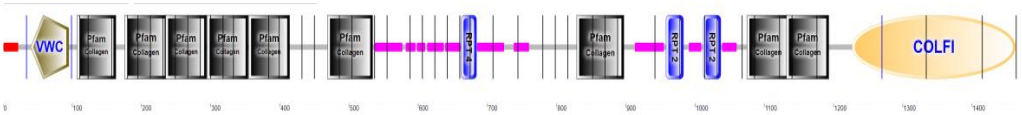
According to Figure 3, SMART protein functional region analysis, all collagens have a C-terminal COLFI recognition region, which is a fibrillar collagen terminal recognition region, and this region is exceptionally important for collagen assembly, and is the main marker for this protein as a collagen recognition family. Some collagens, also have the platelet-responsive protein N-terminal region TSPN, showing that this part of collagen, can bind to platelet-responsive proteins. Overall, collagen evolution still tends to be conservative, and the structural domains that should be present, are generally present in each other. Only in the collagen type I α1 chain comparison, there are more GXXG repeat sequences in pig, bovine, and rat, which are not present in these grass carp. In addition, the repetitive GXXG sequences, which seem to be more frequent in cattle and pigs, seem to have more sequences that are similar to the collagen sequences of pigs and cattle, with a higher degree of similarity, but there are no sequences that are exactly the same in pigs and cattle,

which suggests that the electrophoretic profiles of the two, while being extremely similar, are still distinctive to each other.

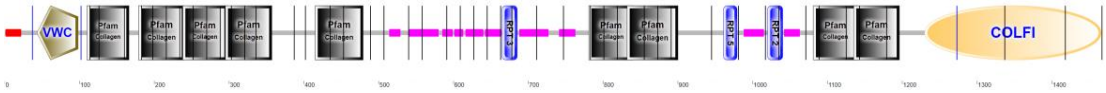
Comparison of collagen type I alpha 1 chains



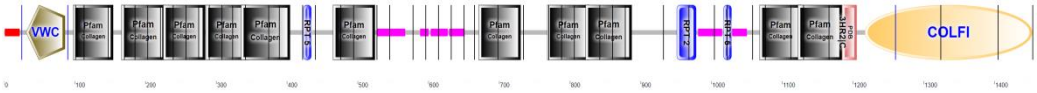
(a) grass carp



(b) cow

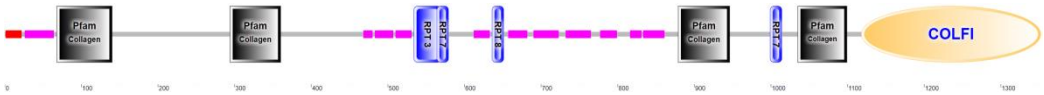


(c) hog

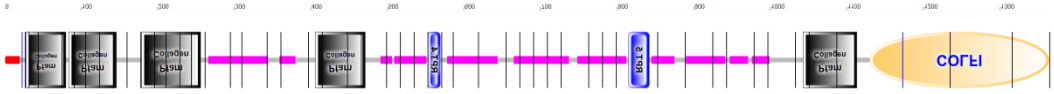


(d) rats

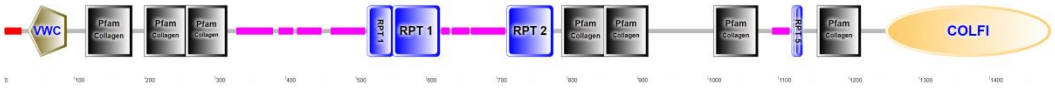
Comparison of collagen type I alpha 2 chains



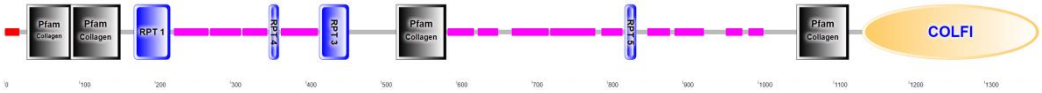
(e) grass carp



(f) cow

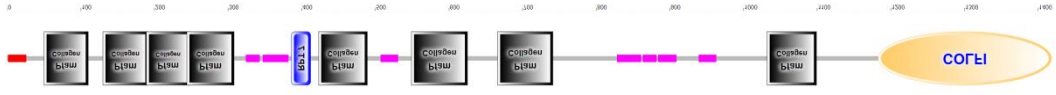


(g) hog

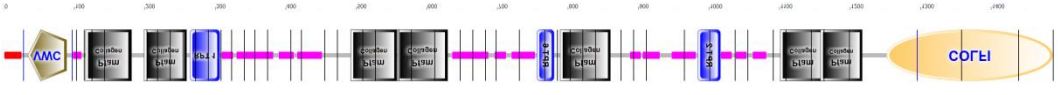


(h) rats

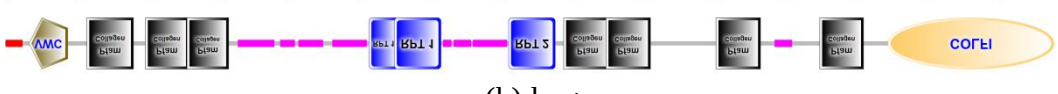
Collagen type II alpha chain



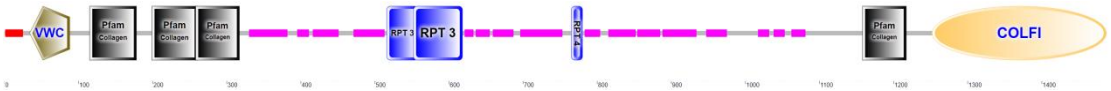
(i) grass carp



(j) cow

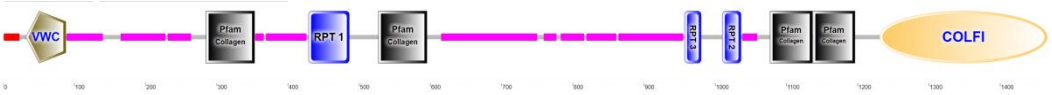


(k) hog

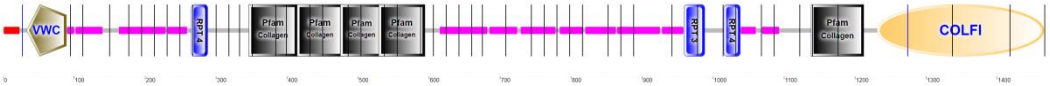


(l) rats

Collagen type III alpha 1 chain

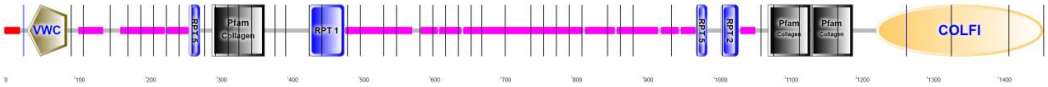


(m) cow



(n) hog

No collagen type III present in aquatic fish

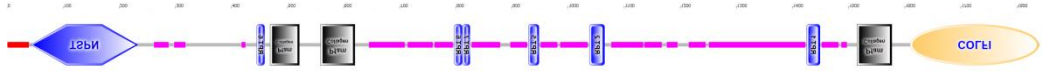


(o) **rats**

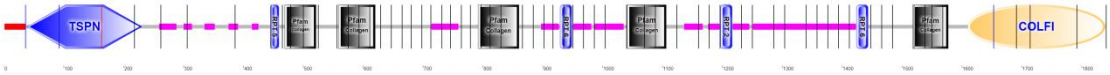
Collagen type V alpha 1 chain

(p) grass carp

(q) cow

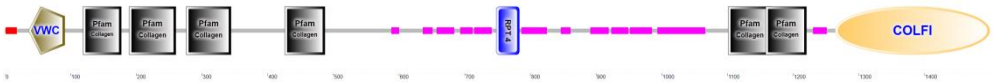


(r) hog

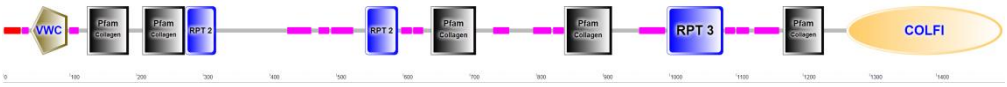


(s) rats

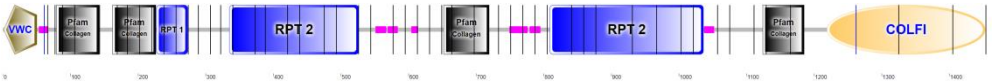
Collagen type V alpha 2 chain



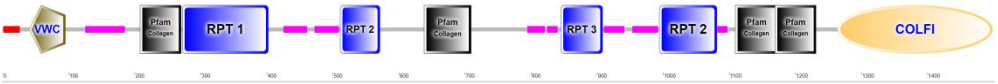
(t) grass carp



(u) cow



(v) hog



(w) rats

Collagen type V alpha 3 chain

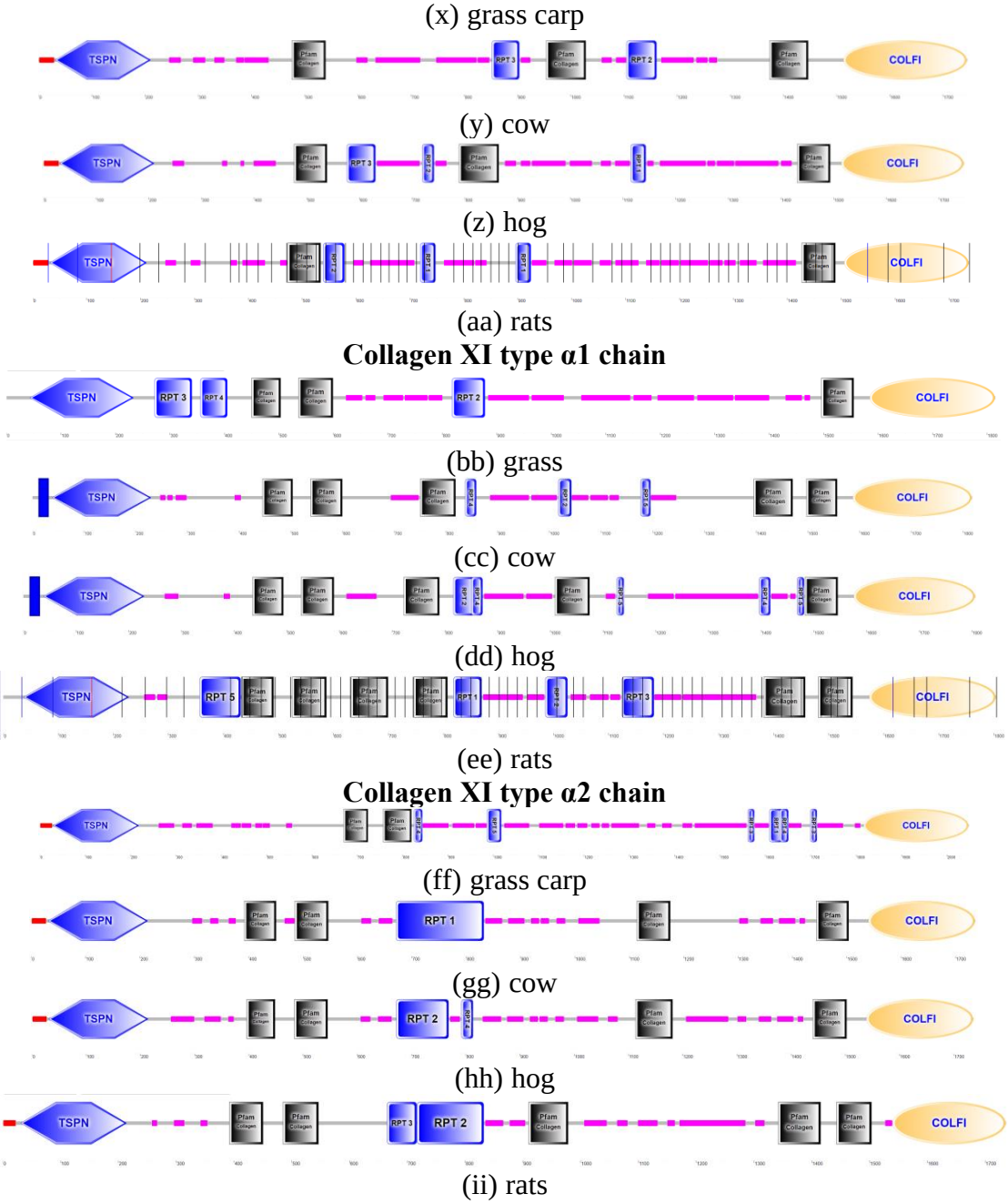


Figure 3 Collagen Smart structure-function domain analysis, each figure scaled to 100 amino acids

4. Discussion

Among the several methods of collagen extraction, enzyme extraction is an effective way to extract collagen, but it is expensive and requires the use of special and expensive enzymes to digest and cleave the collagen. The conditions of enzymatic collagen extraction are more relaxed, only need to consider the appropriate temperature can be carried out, if the use of pepsin treatment, the acidic conditions need to be strictly controlled PH and time of action, otherwise excessive action is easy to damage the high content of protein bands. The enzymatic extraction of collagen during the extraction process allows for a better preservation of the collagen bands after electrophoresis, and is therefore a highly recommended method for the detection of collagen products in this study.

Collagen is a very important tissue structural and functional protein in living organisms. Collagen plays an extremely important role in both tissue construction and function carrying in living organisms. Therefore, the results of SMART function prediction suggest that the sequences of collagen proteins are more conserved with each other [17]. The collagen of the fish grass carp, only a few sites show partial specificity different from pig, bovine collagen, and mouse collagen, and there are more GXXG repeats in rat, pig, and bovine collagen, which are all α-helical, suggesting that a higher degree of fibrillization at this site may result in a higher storage of elastic energy[18]. This potentially hints at the elastic demand of rat, pig and bovine collagens as terrestrial animals for support of the fibrotic solid phase of the corresponding terrestrial life.

Due to the wide distribution of collagen, the evolutionary development of the collagen family should theoretically reflect the evolutionary process of biological organization systems [19]. The use of collagen as a molecular marker of biological evolution has opened the way to the field of molecular evolutionary studies from the field of collagen protein molecular structure and composition by electrophoresis and other methods. In this study, we determined the kinship and species characteristics of grass carp skin, rat tail, pig tendon and cow tendon by measuring the electrophoretic bands of collagen extracted enzymatically from them and analyzing them in comparison with each other. In this study, it was found that the collagen from grass carp had both different and identical bands to that of rat, bovine and porcine collagen. The identical spectral bands suggest that they are properties of collagen. The different bands suggest that the evolution of fish and mammals is different; the results of collagen electrophoresis bands between rats, pigs and cows, especially between pigs and cows, are very similar, and all are typical collagen electrophoresis bands of mammals; while the electrophoresis bands of fish are more different from those of mammals.

Since the collagen sources used in this study were all commercially purchased products from minced animal limbs, they can only be used as a reference for future studies on obtaining collagen sources from a wider range of species. This study also confirms that enzymatic digestion followed by SDS-PAGE electrophoretic analysis can quickly and effectively identify the product quality and source of collagen, which is worthy of further promotion.

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