EXTRACTION, PURIFICATION AND DISTRIBUTION OF FISH COLLAGEN PEPTIDES FOR THE HUMAN HEALTH CARE SYSTEM

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Abstracts

Utilization of fish by bioconversion into high grade products would partially reduce pollution and economic cost associated with treating fish processing waste, fish skill is an abundant supply of gelatin and collagen which can be hydrolyzed to produce bioactive peptides of 2-20 amino acid sequence. Bioactivity and peptides purified form fish with acts as a physical barrier and chemical barrier through antimicrobial peptide innate immune action and other functional peptides. Small peptides which are based on the amino acid composition and sequence.

Collagen is fibrous present which s dominant in the connective tissue and animals it has a wide range of applications in the food, pharmaceutical, cosmetic industries.

There is a growing interest in the extraction process of collagen and its derivatives due to the growing tendency to all this protein to replace synthetic agents in various industrial process.

Key words: Peptides, bioactivity, antioxidant, antihypertensive antimicrobial, antialzhmer's.

Introduction

Collagen is a triple helix built from the amino acid chains. Which forms strong fibers and provide the body with structure. Collagen is the human body's host abundant protein. Nothing u around 30% of total protein content. It ensures the integrity elasticity and strength of our body's connective tissues and thus maintaining the form and function of our thin cartilage and bones.

Different cells in our body are responsible for the production of collagen. The cells are specific amino acids as building blocks for the long chains that are wound together to the large collagen tripe helix several are then organized into the strong fiber that the ability to withstand forces.

Skin 

Collagen Constitutes 70% OF THE skins dry mass content. A key component of the thin's structure. Collagen fiber provide the infrastructure for elastin. which maintain think elasticity and for hyluronic acid to trap moisture.

Tendons 

Tendons are strong fibrous connective tissue that connect muscle to bones, during muscle contraction the tendons role is to transmit force and withstand tension.

Tendons contain 85% collagen type I and also proteoglycans.

Joint cartilage 

Joint cartilage is made up of cellular building blocks (Chondrocytes) which produce an extracellular matrix. Consisting of collagen and boteogycans (Mainly aggrecan). Collagen Fibers make up 70% of cartilage and are responsible for its structure and strength while proteglycans serve as lubricant to the joint.

Bones 

Representing around 90% of organic bone mass. Collagen provides the structural frame work on which calcium and other minerals are anchored. Collagen fibers also provide bone flexibility.

Muscles

The loss of collagen by ageing means a gradually less of it in the connective tissue that bands mass fibers into a strong and functioning muscle. Aging is linked to decrease muscle strength and function which aspects our balance giant. And over all mobility.

What is collagen powder?

There are more than a dozen types of collagen each compared of different "Peptides". Or amino acids. Different types form skin and tendons as opposed to cartilage. Figuring out which may help your health has proved tricky (More one that in a minute). Also supplements containing collagen very a ton.

Most collagen peptides powder on the market contain a hydrolyzed type-I collagen extracted from hidel bones or fish scales.

Hydrolyzed simply means that the amino acids chain have been broken down into smaller unit. A process that allows the powder to dissolve in both hot and cold liquids.

What are the benefits of collagen powder?

The resource on the side effects and potential of collagen supplements is ongoing. But here's what we know right now about the potential upside.

Right now the most complete research focuses on joint health hoping back to at least the early 90's the truth about collagen supplements and their potential health benefits.

The truth about collagen supplements and their potential health benefits

Quickly and recently amino acids have become big business. Where there your shopping for a collagen supplements. Bone broth or even more meat and dairy foods. The different amino acids that made up these proteins are what your ultimately being and ingesting. Says **mark Moyad M.D** director of preventative and alternative medicine at the University of Michigan.

Your body was amino acids to build muscle. bone cartilage. Skin, hair, connective tissue and much more, There are many different types of amino acids. But the most abundant kind in your body make up collagen. Collagen is the main structural protein that forms your connective tissues and skin.

Since your body's collagen production declines as you age and you need adequate collagen for strong bones, joints and skin. Adding more collagen to your diet sound like No-Brainer. That's why many supplements makers have started selling collagen powder

and pills. which **Dr.moyad** Says are made mostly from animals parts like Fish Scales or cow bones or skin.

MATERIALS AND METHOD

The hydrogenous mixture of fish scales obtained for the experimental procedure to extract the collagen powder form it. the fish scale is continuously washed interning tap water ad distilled water. The fish scale is when completely demineralized when it will placed in the EDTA (Ethylenediamine tetra acetic acid) solution for about 2 days (48 hours).

After 48 hours and demineralization wash the scales complete around 3 times using distilled water. Demineralized scales were digested with 0.5 molar acetic acid for a period of 48 hours.

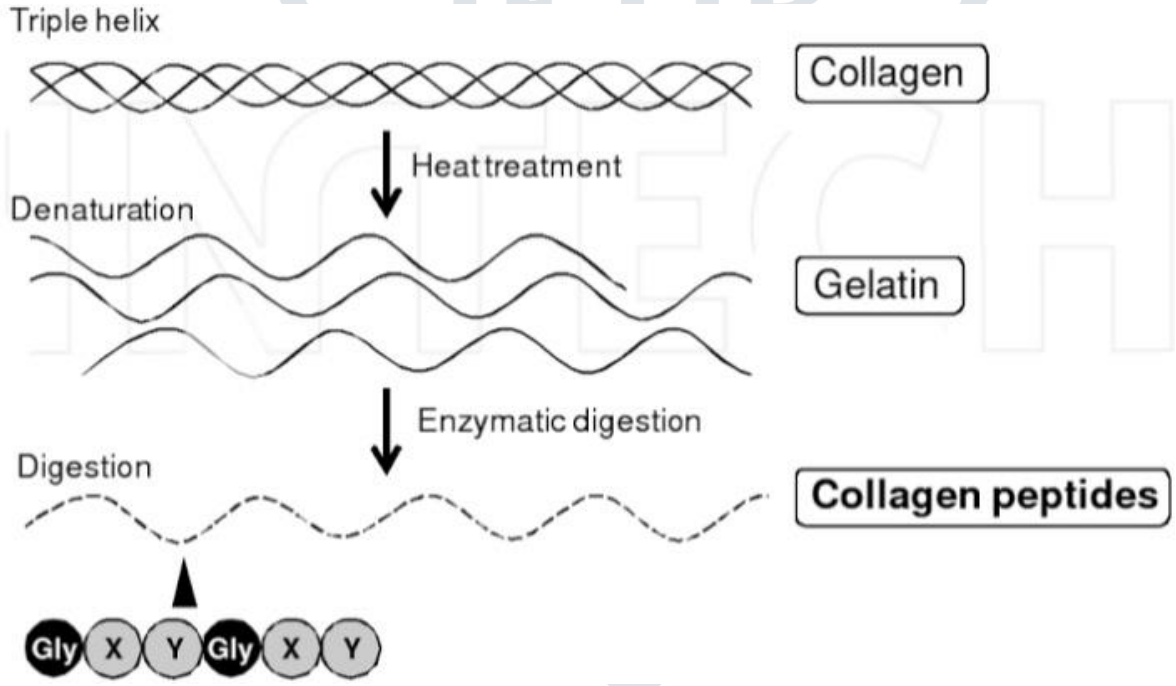
(Demineralization process involves removal of minerals by means of ion exchange resins).

After this period the samples are centrifuged for about 15 minutes at 10,000 rpm after centrifugation the pellet are resolubilize in 0.5 molar acetic acid and dialized using a membrane for 48 hours. After the process of dialization the sample were place freeze dry for 2 day after freeze day. **The some of phases of freeze drying process**

1. Freezing liquid – solid conversion of the product.
2. Primary drying – removal of the frozen solvent by sublimation
3. Secondary drying – removal of unfrozen by diffusion and desorption.
4. After the fraze dry the primary product of collagen power were obtained in this process which compose of different peptides or amino acids. Which will be a more beneficial product in the medical health science and biotechnology prospective.
5. Collagen peptides are easily digestible, cold soluble and highly bioactive sources of collagen.

Although collagen peptides are not precisely the same as gelatin. Both gelatin and collagen peptide originate from collagen and are proteins made from amino acids. Collagen peptide is derived from the enzymatic hydrolysis of collagen.

Grade	Form	Solubility	Absorption & digestibility	Application examples
Native collagen		Insoluble	None	Medical materials, collagen casings
Gelatin		Medium	Low	Gelatin desserts, confectionery
Collagen peptides		High	High	Dietary supplements, functional foods



Collagen Extracting Process

Collagen can be basically obtained by chemical hydrololysis and enzymatic hydrolysis (Zavareze Et el, 2009). chemical hydrolysis is more commonly used in industry. But biological processed that are the addition of enzymes are more promising when products with high nutritional value and improved functionality are required (Martins et al 2009). More over enzymatic processes time. But they are more expensive. To extract collagen, it is necessary to remove numerous covalent intra and intermolecular cross-links.

Which primarily involves residues of lysine and hydroxyl-lysine. Ester bonds and other bonds with saccharide. all of which matches the process quite complex.

Before the collagen can be extracted a pretreatment is performed using an acid or alkaline process. Which varies according to the origin of the raw material. The pre-treatment is used to remove non-collagenous substance and to obtain higher yields in the process. The most commonly used extraction method are based in the solubility of collagen in neutral saline solutions, acid solution and acidic solution with added enzymes.

Pre-Treatment

Due to the nature of the cross-linked collagen that is present in the connective tissue of animals if dissolved very slowly. Even in joining water as a result a mild chemical treatment is necessary to break these cross-links before extraction. To this end. Diluted acids and bases are employed. And the collagens subjected to partial hydrolysis which maintains the collagen chains intact but the cross links are cleaved.

In the acidic from of pre-treatment the raw materials is immersed throughout the material. As the solution penetrates the structure of the skin at t a controlled temperature it swells to two or three times its initial volume and the cleavage of the non covalent inter and intra-molecular bonds occurs.

The acidic process is more suitable for more fragile row materials with less intertwined collagen fibers. Such as porcine and fish skins.

The alkaline process consists of treating the raw material with a basic solution typically sodium hydroxide (NaOH) for a period that can take from a few days to several weeks. This process is used for thicker materials that required a more aggressive penetration by the treatment agents. Such as bovine ossein or having. NaOH and $CaCoH_2$ are often used for pre-treatment.

But NaOH is better for pre-treating skins because it comes significant leveling. Which facilities the extraction of collagen by increasing the transfer rate of the mass in the tissue matrix.

A study by Lin et. al. (2015) evaluated the effect of alkaline pre-treatment on the extraction of acid soluble collagen (ASC) from the skin of grass carp (Ctenophrayngodon idella). Concentration of NaOH from 0.05 to 0.1m were effective in removing non-

colagenous proteins without losing the ASC and structures modifications at temperature of 4,10,15 and 20^0C. However 0.2 and 0.5 in NaOH resulted caused a significant loss of ASC and 0.5m NaOH resulted by structural modificationinthe collagen at 15 and 20^0C. in addition may also be used to cleave the cross linked bonds to obtain products with different characteristics.

Chemical Hydrolysis

In the extraction of collagen which is soluble in salt, neutral saline solutions are used, such as sodium chloride (NaCL). Tril-HCl (Tri Hydroxymethyl) amino methane hydrochloride, phosphate or citrates. Caution is required in this process in order to control the concentration of salt. But considering that the majority of college molecules are cross linked the use of this method is limited.

For the extraction of acid-soluble collagen. The pre treated material is added to the acid solution. Usually 0.5m acetic acid. And maintained for 24 to 72 hours under constant stirring at 4^0C depending on the raw material.

After the extraction stage a filtering is performed to separate the supernatant (residue) from the collagen, which is the liquid phase, to maintain collagen powder. The filtrate is usually subjected to precipitation with NaCL. The precipitated is then collected by centrifugation and subsequently redissolved in a minimum volume of 0.5m acetic acid and then dialyzed in 0.1 acetic acid for 2 days and distilled water for 2 days with replacement of the solution on average every 12 hours.

Preparation of bioactive peptides form fish skin

Fish protein hydrolysates contain peptide of 2-20 amino acid sequences after hydrolysis and three peptides usually have biological activity, several extraction methods are utilized to liberate bioactive peptide form the parent protein. And three include acid alkaline hydrolysis. Extracting collagen by using acidic or alkaline reagent. Enzymatic hydrolysis using enzymes to hydrolyze fish skin. And fermataton method. Using microorganisms as a source of the enzymes (Hvang et. at. 2015).

Enzymatic Hydrolysis

Enzymatic hydrolysis is the best way to hydrolyze fish skin without losing nutritional value (Huang et. al. 2015). The method is preferred especially in the food and pharmaceutical industries because the hydrolysis process does not leave residual industrial because the hydrolysis process does not leave residual organic solvent s or toxic chemical in its products. Steps in enzymatic hydrolysis involve substrate preparation choice of the right enzyme. Measuring the extent of enzymatic hydrolysis, homogenization and meting to inactive endogenous enzymes hydrolysis and termination of the enzymatic reaction. Commercial enzyme such as alcales .trypsin, pepsin, papain, Pancreatin, and thermodysine are employed in the enzymatic hydrolysis. Conditions like enzyme concentration pH time, hydrolysis, enzyme concentration pH and temperature have to be well monitored and maintained during hydrolysis. enzyme with the type of enzyme used, enzyme concentration of 0.01-5.00% (w/w) and pH range of 1.5-11 have been documented (Halim et. at. 2016) black – Barred half beak gelatin was dissolved with Distilled water and subjected to enzymatic hydrolysis with an enzyme / substrate ration of 30:1, pH 10.0 and 50^0C the enzymatic activity was evaluated by a method described by (Kembhavi et. al. 1993) using casein as substrate. The gelatin solution was equilibrated for 30 min before the enzyme addition. The pH was maintained by addition of 2N NaOH, and after 3h. the enzymes were inactivated by heating the solution at 95^0C for 20 min. extracted of pepsin soluble collagen (PSC) from fish skin was performed by (Mahaboob 2014). Undissolved residue obtained after acid soluble collagen (ASC) extraction was utilized for the PSC extraction as described. (Singh Et. al. 2011).

Acid-alkaline hydrolysis.

During fish hydrolysis by acid-alkaline hydrolysis contain amino acids i.e. tryptophan, serine and threonine, can be destroyed at high pH. Therefore, the pH and temperature of the hydrolysates must be closely observed during the hydrolysis process collagen extraction for fish skin by acid alkaline hydrolysis treatment of pre-cleaned skin samples with an alkali (NaoH) is an initial extraction step. The step's followed by continuous stirring at a controlled temperature for a set time.

The procedure is repeated about 3 times and it is carried out with an aim to remove non-collage nous proteins acid (Hcl). After acid (HCl). After acid alkali treatment the skin

was washed to neutralize the pH and further extraction carried out with distilled water at 65^0C for 4 hrs.

At using butly alcohol for 24-48hr with gently string and change of solution energy 80h. The resultant matters was then subjected to acid treatment with acetic acid aws then subjected to acid treatment with acetic acid for 24hr with gently stirring collagen was extracted from fish skin. Scale and bone using a procedure described as follows (Wang et. al 2008). The collagen was extracted were centrifuged at 20,000 g for 1hr at 4^0C and the extraction step was repeated using the obtained residue followed by centrifugation under the same conditions the suprematurants of the two extracts were combined and precipitated by the addition of NaCL to a final concentration of 0.9m and centrifuged at 2500g for 0.5h to obtain a precipitate that was dialyzed for 48h against 10 volumes of 0.1m acetic acid and distilled water. Respectively where were changed every 8h. Before being lyophilized, anti microbial peptides were purified from winter flonder epidermis and mucus extracts (Cole et. al 1997). The mucus was obtained from the skin by scrapping and further subjected to homogenization in a solution of 50ml of 0.2m sodium acetate. 0.2% triton X-100 and 1M phenyl methyl sulfonl fluoride. The homogenate was centrifuged for 20 min at 20,000g and the resultant supernatant was further purified.

Fermentation

Fermentation is considered a more natural method and protein especially in East Asian countries as a traditional preservation method. Fermentation not only enhances the flavor during the fermentation process. Bioactive peptides are released by the action of both microorganisms and endogenous protocol tic enzymes. Several studies have demonstrated the shins paste. Shrins by – products. Squid micro and a variety of traditional fermented form products. Majumdar et al. 2016 traditional fermented fill product of northeast India. A combination of both fatty acids. (Eicolapentaenoic, docosanexanoic. Arachidonic, linolenic, and kholeic acid) and proteins or of MW (Molecular weight) range between 45 and 29 kDa and 45 and 6kDa respectively were reported to be present in the fermented fish product. Hydloysates were prepared form turbot skin by utilization of the fermentation method using 3 microorganisms. i.e., saccharmoycer cervisia. Aspergillus oryzae. And streptocellus thermophilus.

Purification of Peptides

The biological activity of peptide is determined by properties like molecular weight, charge, and hydlophoicity. Therefore, peptide are purified through a multi step purification process based on such properties purification based on molecular weight employs method like ultra filtration (UF) nano filtration (NF) and gel filtration (GF) ion exchange chromatography (IEC) is used to fractionate peptides based on their net charge fractioned peptides are then further purified using technologies like reverse-phase HPLC which separates compounds based on hydrophobicity and hydrophilicity (Conlon 2007). Peptide sequences of the most active tractions from HPLC analysis are then analyzed and identified using mass spectrometry methods like matrix-assisted laser deionization time of flight (Maldi-TOF). Electro spray ionization mass (ESI) matrix-assistdsss laser desorption / ionization mass spectrometry (MALDI-MS) etc. (Bernardini et. al. 2011).

Anti Alzheimer's and neuro protective activity

Alzheimer's disease is a kind of neurodegenerative disease characterized by progressive loss of nervous. The prevalence of such degenerative neuro-disease has increased with an increase in life expectancy especially as seen in developed countries (Choi and Choi 2015). Anti-Alzheimer's disease activity is profiled using β-secretace inhibitory activity. The enzyme β-secretace along with another enzyme r-lecretase generate a peptide amyloid –β(Aβ) through endo-proteolytic reactions of the amyloid precursor protein (APP) (Choi and Choi 2015) apoliporotein enhance the breakdown of beta-amloid, however an iso form of apoliprotein, APOEU, ineffectively breakdown beta-amyloid and leads to an excess amyloid build up in the brain .the peptide Aβ molecules can aggregate to form flexible soble oligomers, some of which turn out misfloded. These misfiled oilogmers can induce other Aβ molecules to also take the misfold ooigomeric form.

Anti-Alzheimer's and neuroprotecive activity of fish skin hydrolysates is summarized. In table 1 Aβ – secretase inhibitor peptide was purified form skate skin hydrloysate.

The peptide was purified forma neutrase hydrloysate of skate skin on a sephadex G-25 column and with reversed-phase HPLC, the peptide sequence was determined to be QGYRPLR GPEFL and showed β-secretace inhibitory activity with an IC50 value of 24.20mm. The neuroprotective effect of protein hydrolysates with antioxidant activity from grass carp (ctenopharyngodon idella) skin was demonstrated (cai et al. 2015). The

hydrolysates at the degree of hydrolysis DH5,DH10 and DH15 Showed the most significant neuroprotecive effect on 6-OHDA induced neurotoxicity in MES 23.5 salmon (oncorhyndrus keta) skin enzymatic hydroloysate showed learning and memory enhancement in mice (Pei et. al. 2010). Oxidative streets was alleviated asoptolic nervous reduced. And brain – derived neurotohoic factor (BDNF) expression was uperergulated by treatment groups compared with the control group. Similarly another study showed that salmon skin collagen peptides reduced oxidative damage and acetylochlinesterace (ACHE) it increased phosphorylated. cAMP response element binding protein (P-CREB) and BDNF expression in mice (xu et. et. 2015).

Table 1

Anti – Alzheimer's and neuroprotective activity of peptide purified from fish skin

Activity of mechanism	Species	Peptide sequence	Reference
β-secretale inhibitory	Skate (raja Kenojei)	PGYRPLRGPEFL	Lee et al. (2015)
Anit – Acetylchlinesterase	Salmon concorhyncus (Keta)	-	xv et al. (2015)
Neuroprotection	Grass carp (Ctnophyrngale)	-	cai et al. (2015)
Learning and memory	Salmon (oncorhynchus Keta)	-	pei el al. (2010)

Other Biological Activity

Other biological activity including antihyperglycemic MMP inhibitory activities and adipogenic regulatory have been demonstrated usingfish skin as shown in table 2. Anti hyperglycemic activity of fish skin was in evaluated using dipeptidyl peptidase IV (DPP-IV) inhibitory assay. Steelhead (oncorhynchus mykiss) skin gelatin hydrloysates were prepared. And the hydrolysate of 4% papain had highest DDP-IV inhibitory activity 40-45% (Chenng and Li-chan 2017). The hydrolysates were purified with ultrafiltrationto obtain fraction of less than 3kDa. Two fractions showed 42% and 44% DDP-IV inhibitory activity showing that the activity was not influenced by ultra filtration as the value of the fraction and the whole hydrolysis were similar.

In a similar study the DDP-IV inhibitors and glucagon like peptide-1(GlP-1) Stimulating activity of fish skiing gelatin from various warm-and cool-water fish skins were animated and compared (Wang et. al. 2015) regulate revealed that the DPP-IV inhibitory activity of gelatin hydrloysate from warn water fish was greater than that cold water fish habitat and tilapia skin gelatin hydrolysis (HSGH and TSGH) fraction at a cut off of <1.5 kDA UF were used for peptides leghen a identification and to compare the in vivo aythipeglycemic effects. MS/MS spectra analysis revealed amino acid. Sequences of 6 active peptides as SPGSSGPQGFTG, GPVGPAGNPGANGLN, PPGPTGPRGQPGNIGF, IPGDPGPPGPPGRP, LPGERGEPGAPGP, AND GPKGDRGLPERPPGRDGM. All these peptide possed the amino acid prokine as the second N-terminal residue. Mover eve it has been reported that peptide with DDP-IV inhibitory activity has amino acids proline, tryptophya, alanine, valine, lysine, and aspartate as the second N-terminal residue in their sequence.

Fish skin hydrolysates have also been demonstrated to show MMP-inhibitory activity and thus have great potential use as cosmeceutical. Two active peptide form code skin gelatin hydloysate (CGH) with anti-photo ageing activity were identified (Le et. al. 2017) the peptides were purified from CGH by ion exchange chromatography and RP-HPLC.

The anti – photo aging effect of a peptide LMWCP purified form cat fish skin (Pangasius Hypophthalmus) was evaluated both on animal modus and in clinical trial. The peptide down regulated the expression of MMP-3 and MMP-13 unlike it upregualte the expression of MMP-2 and MMP-9 (Pyun et al. 2012). In the clinical trial results. Treatment grows reciwing a daily oral dosage of 1000mg of LMWCP for 12 weeks showed significantly improved skiing and less wrinkling in comparison with the place group (Kim et al. 2018).

Last but not least, fish skin has also been demonstrated to have adipogenic regulatory activity. The effect of subcritical water-hydrolozed fish collagen peptide (SWFCP) from tuna skin on the protein levels of the master adipogenic transcription factors C/EBP and PPAR was investigated (Lee et. al. 2017). This was done with the aims of evaluating the underlying inhibitory mechanisms of SWFCP in the adipogenic differentiation of 3T3-L1 pre-adiopolytes. Results revealed that sWFCP down regulated the expression of the key adipogenic target gene and transcription factors in3T3-L1 pre-adipolytes exposed to MDI.

After 8 days of incubation of 3T3-L1 cells with 1mm dexamethasons ad 1mg /1ml insulin (MDI) and SWFCP the expression level of C/EBP and PPAR protein were greatly reduced compared with cells stimulated with MDI alone.

SWFCP was also shown to down regulate the expression of a P2 an adipogenic target gene. Hence inhibiting adipogenic differentiation. Further more, SWFCP reduced lipogenesis inhepatcytes. This was demonstrated by the use of Palmitate-induced intracellular lipid.

Vacuole accumulation visualized by Nile red staining. The palmitate induced intracellular lipid vacuole accumulation was gretlyh reduced in the presence of 1mg/ml SWFCP. SWFCP significantly affected other obesity related factors like low serum cholesterol. Low serium triglyceride and low-density lipoprotein, high serum high-density lipoorten levels. And reduced dize of epididymal adipocytes.

Table 2

Antihyperglycemic and MMP inhibitory activity of peptides purified form fish skin

Mechanism / activity	Species	Peptide sequence	Reference
Antihyperglycemic	Halibut (Hippoglossus stenmlepis)	SPGSSGPQGFTG, GPVGPAGNPGANGLN, PPGPTGPRGQPGNIGF,	Wang et al. (2015)
	Tilapia (coreochromils niloticas)	IPGDPGPPGPPGRP, LPGERGEPGAPGP, GPKGDRGLPERPPGRDGM	
MMP inhibitor activity	Cod Tilapia (oreochromis	ELGPSGGRGKPGKGDAGPK, GFSGLDGAKGDLSGYGP	Lu et. al. (2017)

	niloticus) Sutchi cat fish (Pangasius hypophthalmus)	LMWCP	sun et. al (2013 pyun et. al. 2012)

Collagen peptide and gelatin market over view

The global collagen peptide and gelatin market size was estimated to be $3,727.34 (dollar) million in 2017. And expected to reach $6,729.00million by 2025. Registering a (AGR compound annual growth rate) of 7.8% from 2018 to 2025.

Collagen peptide market overview

With respective to the gelatin the collagen peptides are versatile source of protein and an important element of health nutrition.

One of the major drives for this market is demand or collagen peptide in nutrition – based products.

Collagen peptide is extensively used in the nutrition – based food industry due to its high protein content. Collagen peptide consists of many amino acids which are the building blocks of protein. It helps in reducing health-related risks such as osteoporosis, juvenile Blindness, rheumatoid arthritis and cancer. Collagen peptide is commonly used in various nutritional supplements. Anti-inflamation supplements. And joint cartilage supplements because of the high concentration of amino acid in it.

According to this study over the next five years the collagen peptides market will register a 4.4% CAGR (Compound Annual Growth Rate) in terms of revenue.

The global market size will reach $3820 million dollar by 2024. From $2950 million dollar in 2020 in particular this report present is the global market share (Sales and revenue).

To study and analyze the global collagen peptides consumption (Value and Volume) by products type.

The analysis of collagen peptides with respect to individual growth trends future prospects and their contribution to the total market.

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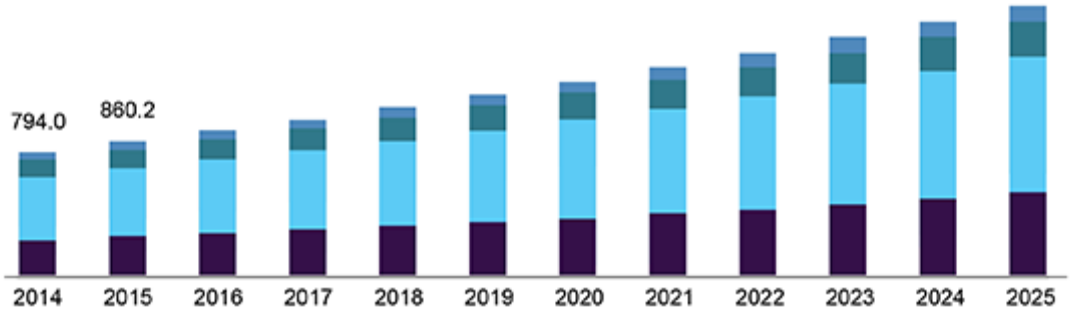

Graph Line of Market Potential

Sample Product Collagen Powder in the present market