

Recent advances in Crocodile-derived Bioactive Peptides

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Abstract Bioactive peptides are a group of biological molecules derived from animals, plants, or microorganisms, which can naturally occur or be obtained through proteolytic cleavage of proteins. Increasingly, these peptides have been developed into functional foods, specialized medical foods, and pharmaceuticals. Crocodile-derived bioactive peptides have gained consumer favor over the past decade. In this review, we summarize the sources, the production methods, and the applications of these peptides including antibacterial, antioxidant, anti-aging, anti-tumor, and anti-inflammatory effects, and discuss the prospects for the future development of the crocodile peptide industry, which provides a reference for researchers interested in biomedicine, functional foods, bioactive peptides, and the crocodile industry.

Keywords: Bioactive peptide, Crocodile, Antibacterial, Antioxidant, Anti-aging

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1. Introduction

Bioactive peptides are primarily isolated from diverse sources, including bacteria, fungi, plants, and animals. They typically consist of simple linear chains of amino acids, ranging from a few to several hundred residues, but some of them also exist in cyclic forms with more complex structures [1]. Most bioactive peptides are considered specific protein fragments that offer health benefits to humans by exerting various biological activities, thereby contributing to overall wellness and potentially preventing diseases [2]. Recognized as a new generation of bioactive regulators, these peptides play a crucial role in preventing food oxidation and enhancing disease management. Their unique properties make them valuable for promoting health and extending the shelf life of food products [3]. They primarily affect the cardiovascular, nervous, digestive, and immune systems, with large dependent on the specific amino acid residues. Therefore, the study of the structure and function of various peptides is essential for promoting human health and enhancing immunity [4].

The crocodile is one of the oldest living species on Earth, appearing as early as 200 million years ago [5]. A total of 23 species of crocodilians have been identified, divided into two families: Alligatoridae and Crocodylidae.

The Alligatoridae family includes the genera *Alligator*, *Caiman*, *Paleosuchus*, and *Melanosuchus*, while Crocodylidae comprises *Gavialis*, *Tomistoma*, *Mecistops*, *Osteolaemus*, and *Crocodylus*, the largest genus with 11 species [6]. Most crocodile species are found in Africa, Asia, North and South America, as well as in the humid, swampy tropical regions of Australia [7,8]. Crocodiles are fierce, egg-laying carnivores that feed on fish, waterfowl, deer, and other prey. Unfortunately, their wild populations have significantly declined due to habitat loss, hunting, and human persecution [5]. They live for extended periods in bacteria-rich, murky environments often contaminated with heavy metals and frequently feed on carrion. Despite these harsh conditions, they rarely fall ill [9], leading to speculation that crocodiles may possess potent antibacterial properties.

In recent years, increasing attention has been directed toward studying the potential applications of materials derived from crocodiles. Substances obtained from crocodile organ lysates have demonstrated a range of bioactive properties, such as anti-amoebic [9], wound healing [10], treatment of anemia [11], antioxidant [12], anti-tumor [13], anti-inflammatory [14], anti-aging [15], and antibacterial effects [16]. However, the use of crocodile-derived products is still in its early stages. This paper reviews recent advancements in research on crocodile peptides and the development of crocodile peptide-based products for medical and health applications.

2. Crocodile Peptides and their Preparation

The use of bioactive peptides has long been recognized as a promising strategy due to their favorable biosafety profiles and their medicinal and nutritional properties [17]. Bioactive peptides are typically composed of multiple amino acid residues, many of which remain concealed within the intact protein structure [18]. These peptides exhibit a range of biological effects, including blood pressure reduction, anti-obesity properties, wound healing, and antioxidant activity [19]. Investigations in animal models have demonstrated the significant potential of these peptides as health-promoting agents in both food and pharmaceutical applications [20].

Bioactive peptides are typically obtained through various methods such as acid and alkaline hydrolysis, fermentation, chemical synthesis, genetic engineering, and enzymatic hydrolysis. Some peptides are redesigned based on existing proteins or enzymes [21]. Fragments derived from hydrolyzed crocodile hemoglobin using hydrogen bromide exhibit antibacterial properties and promote wound healing [22]. Acid-base hydrolysis involves breaking down proteins into peptide fragments. Under conventional acid hydrolysis conditions, the recovery rate of amino acids is often low, particularly for hydrophobic proteins. When sequencing these hydrophobic proteins, challenges arise due to their insolubility and the inaccessibility of chemical reagents to the hydrophobic regions [23]. Heidelberg discovered that strong organic acids can effectively hydrolyze hydrophobic peptide bonds. A mixture of concentrated hydrochloric acid and trifluoroacetic acid, when used in the right proportions, was proved more efficient than conventional methods [24].

Chemical synthesis of polypeptides includes liquid and solid-phase methods [25]. Solid-phase synthesis primarily utilizes Fmoc (9-fluorenylmethoxycarbonyl) and Boc (t-butyloxycarbonyl) strategies. In Boc synthesis, t-butyloxycarbonyl serves as the α -amino protection group, removable using trifluoroacetic acid (TFA), while benzyl alcohols are employed for side-chain protection [26]. During synthesis, a Boc amino acid derivative is covalently attached to the resin. The Boc group is then removed using TFA, activating the free amino terminal with triethylamine and dicyclohexylcarbodiimide (DCC). Subsequently, the next amino acid is coupled, and finally, the protective groups are removed [27]. The primary difference between Fmoc and Boc synthesis methods lies in the protective groups used. Fmoc serves as the α -amino protective group, which is removable by alkaline conditions. In Fmoc synthesis, side-chain protection is typically achieved using t-butyloxy groups, removable with TFA. Combining with 90% TFA, p-alkoxybenzyl alcohol resin has an excellent cleaved efficiency, avoid of the strong acid treatment [28]. Advantages of the Fmoc method include convenience, speed, ease of automation, and the ability to synthesize longer peptides. Barksdale et al.,(2016) used the Fmoc chemical method to synthesize the known crocodile peptides [29]. They successfully synthesized three new peptides—Apo5, Apo6, and A1P—using this method and concluded that these peptides

exhibit strong *in vitro* activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

Enzymatic hydrolysis is the most common method for producing bioactive peptides, involving the enzymatic cleavage of peptide bonds in protein molecules [30]. This process breaks down proteins into smaller fragments and amino acids using appropriate proteases [31]. The enzymatic hydrolysis process and its products are controllable and reproducible [32]. Importantly, peptides derived from enzymatic hydrolysis do not diminish the nutritional value of the original protein. The reactions are highly specific and relatively mild, preserving both the nutritional value and functional properties of the resulting peptides [33]. However, enzymatic hydrolysis also has disadvantages such as low efficiency and being time-consuming [34]. Lueangsakulthai et al.,(2021) employed this method to obtain initial products by enzymatic hydrolysis of crocodile white blood cell extracts using trypsin and papain and they found the obtained peptides exhibited excellent antioxidant effects [35].

Fermentation is another method for preparing bioactive peptides by hydrolyzing substrate proteins using proteases produced by microorganisms [36]. Microorganisms can produce a variety of complex enzymes during their metabolic processes, such as proteases that efficiently degrade substrate proteins [37].

The gene recombination expression method can also be used to produce target peptides. The DNA corresponding to some peptides can be introduced into a plasmid vector and then transformed into prokaryotic or eukaryotic strains for expression [38]. This method has a strong expression orientation and a low production cost but poses challenges in extraction, purification, and gene expression research and development [39]. Currently, no studies have used this method for the preparation of crocodile peptides.

Extraction and separation are critical steps in the development of the polypeptide industry. Common methods for extracting and separating polypeptides include salting out, ultrafiltration, gel filtration, isoelectric point precipitation, ion exchange chromatography, affinity chromatography, adsorption chromatography, and gel electrophoresis [40]. These methods have been used to extract crocodile peptides [41]. For example, extracts from crocodile liver were partially purified using ammonium sulfate precipitation to extract the target peptide [42]. In this method, the protein is precipitated with ammonium sulfate, increasing the solubility of the globular protein after salt addition. However, whether through mechanical treatment or solvent-based extraction methods, cell homeostasis is inevitably disrupted, leading to potential protein degradation or instability [43]. Kristinsson et al.,(2000) obtained peptides with antibacterial and antioxidant activities by acid-hydrolyzing crocodile white blood cell extracts [44]. Their findings demonstrated that these peptides effectively inhibited the growth of various pathogenic bacteria while also exhibiting strong antioxidant properties, suggesting their potential applications in food preservation and health supplements. Further studies could explore the mechanisms underlying these activities and the peptides' efficacy *in vivo* [10].

3. Bio-activities of Crocodile-derived Peptides

Recent studies showed that crocodile peptides have a variety of biological activities, such as antibacterial, antioxidant, anti-aging, anti-tumor, and anti-inflammatory. (Figure 1)

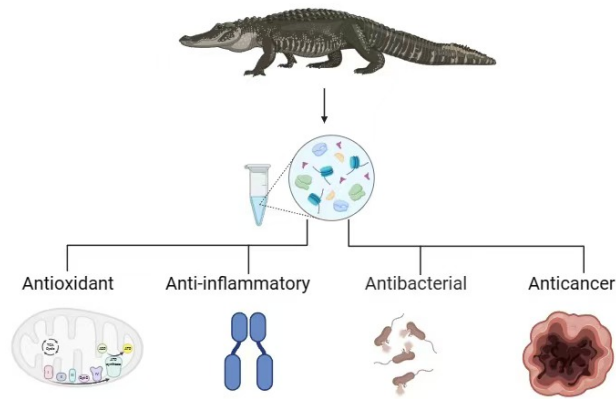


Figure 1. Bioactive role of crocodile peptides

3.1. Anti-bacterial

Because crocodiles inhabit swampy areas rich in resilient microorganisms [9], an increasing number of

antimicrobial peptides have been identified in these animals. The most typical antimicrobial peptides can destroy bacterial cell membranes, leading to cell death [45]. To date, 45 potential antimicrobial peptides have been identified, among which 24 are categorized as cationic antimicrobial peptides [46] (Table 1).

APOC164–88~ A1P394–428 is the American Alligator(*Alligator mississippiensis*) Host Defense-Peptidome, The team evaluated the antibacterial effects of five peptides, all of which exhibited antibacterial activity against various bacteria. Notably, the peptide APOC177-88 has demonstrated a significant activity against *Pseudomonas aeruginosa*. CATH1 to CATH6, derived from the Chinese alligator, are novel antimicrobial peptides exhibiting antibacterial and immunomodulatory effects. Peptides such as Am2HR to HBD3 have been synthesized or characterized from beta-defensins native to crocodiles. Among these, Am23SK monoisomers exhibit an extensive antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella typhimurium*, *Acinetobacter baumannii*, and *Enterobacter cloacae* *in vitro*; concentrations of Am23SK can inhibit the OD₆₀₀ of planktonic bacteria by more than 90%. Additionally, peptides AM-CATH36, AM-CATH28, AM-CATH21, and NA-CATH, derived from the *Mississippi alligator*, have shown remarkable effects against *Acinetobacter baumannii* and *Klebsiella pneumoniae*.

Table 1. Sequences and physicochemical properties of crocodile antimicrobial peptides

Peptide	Sequence	Length (res)	Molecular Weight (Da)	Net Charge	pI
APOC164–88	FSTKTRNWFSEHFKKVKEKLKDTEA	25	3101.65	4	10.0
APOC167–88	KTRNWFSEHFKKVKEKLKDTEA	22	2766.49	4	10.0
FGG398–413	YSLKKTSMKIIPFTRL	16	1925.12	4	10.5
FGG401–413	KKTSMKIIPFTRL	13	1561.94	4	11.3
A1P394–428	PPPVIKFNRPFLMWIVERDTRSILFMGKIVNPKAP	35	4106.28	4	11.0
As-CATH1	RRSGWWNGHKRRRGSGSRHGYSSSTKYGGRKRPRKRPGSGSW LSHDTPHVAPIAKGHVG	59	6630.4	14	12.18
As-CATH2	RRSGWWNGHKRRRGSGTRRGFRSHIAHGGRKGHERIA	37	4334.8	11	12.60
As-CATH3	PAKPKPRPGKLSSTLHLAPGSDGKPRCHYP	31	3535.2	5	10.19
As-CATH4	RRGLFKKLRRKIKKGFKKIFKRLPPVGVGVSIPLAGRR	38	4402.5	15	12.78
As-CATH5	TRRFWKVKVLNGALKIAPFLLG	22	2257.2	6	12.03
As-CATH6	TRWLWLLRGGLKAAGWGIRAHNLNRNQ	26	3044.6	5	12.48
Am2HR	HCKRNDNRNCRHTPCKPAERIIGWCLSTYVCCR	32	3820.48	4.9	-
Am11RR	RCRKGLFCRPKCGOKEHVIGTCPKGLICCR	30	3391.17	6.8	-
Am17RV	RCNLLNGVCRHTLCHSLEKYIGRCHRLNRCCV	33	3828.55	5.0	-
Am23SK	SCRFSGGYCIWNWERCGRSGHFLVALCPFRKRCCCK	34	4070.82	5.8	-
Cp13OK	QCRNNHGHCRRLCFHGEQWIGNCNGRHQHCCK	32	3833.32	4.1	-
HBD3	GIINTLQKYYCRVRGGRCVLSCLPKEEQIGKCASTRGRKCCRR KK	45	5161.2	10.7	-
AM-CATH36	GLFKKLRRKIKKGFKKIFKRLPPIGVGVSIPLAGKR	36	4058.14	13	-
AM-CATH28	KIKKGFKKIFKRLPPIGVGVSIPLAGKR	28	3076.90	9	-
AM-CATH21	GLFKKLRRKIKKGFKKIFKRL	21	2633.37	11	-
NA-CATH	KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	34	4175.26	15	-

This discovery helps explain the long life expectancy and low disease occurrence rates observed in crocodiles, despite their exposure to bacteria-rich environments. Generally, these peptides are characterized by a high biological activity, a low toxicity, and a high specificity [47].

Another natural group of peptides is the host defense peptides, components of the crocodile's innate immune system. These peptides directly combat invading microbial pathogens and play a crucial role in promoting and regulating host immunity [48]. They are characterized by a powerful, a broad-spectrum, and a rapid bacteriostatic activity, and are unlikely to induce bacterial drug resistance.

Crocodiles possess a robust immune system, and extracts from their plasma and white blood cells exhibit broad-spectrum antibacterial effects [49]. It is thought that cationic antimicrobial peptides are the active components responsible for this antibacterial activity [50]. The cationic antibiotics cOT2 and sOT2 in crocodiles have been extended through their amino acid sequences to form new peptides, which have demonstrated destructive effects on intact bacterial cells [16]. These two peptides were isolated from reptile egg transferrin via chromatographic purification and characterized using mass spectrometry and N-terminal sequencing. To enhance antibacterial efficacy, two new peptides cOT2(+6) and sOT2(+5) were designed and synthesized by extending the primary amino acid sequences of cOT1 and sOT1. cOT2 and sOT2 can induce various morphological changes in bacterial cells, with these effects increasing at higher peptide concentrations. Additionally, Kommanee et al.,(2007) purified an antibacterial substance, a lysozyme-like protein, from crocodile white blood cell extracts. They found that this substance exhibited antibacterial activity against *Pseudomonas aeruginosa* ATCC 2785 and *Vibrio cholera* [51]. Another study demonstrated the antibacterial and bactericidal effects of undiluted crocodile plasma against both Gram-positive and Gram-negative bacteria [52]. Crocodiles also possess an innate immune component known as β -defensin. Researchers synthesized and characterized this peptide, and the results indicated that the newly formed peptides exhibit antibacterial activities against *Salmonella typhi*, *Staphylococcus aureus*, and other pathogens [53]. Crocodile defensin CpoBD13 exhibits potent antifungal activity mediated by pH-dependent membrane targeting [54]. It is the most abundant and widely expressed β -defensin among all vertebrates, capable of directly targeting negatively charged phospholipids. Li et al.,(2023) investigated the antibacterial mechanism of *Alligator sinensis cathelicidins* against Gram-negative bacteria. Through molecular dynamics simulations, it was found that the infiltration of AS4 hydrophobic residues into the bacterial membrane disrupted lipid accumulation, causing an imbalance in tension between the two leaflets and ultimately leading to the destruction of the membrane structure [55].

To enhance antimicrobial activity, the proposed RN15 peptide was modified. They employed template and physicochemical methods to design antimicrobial peptides and predicted RN15 derivative peptides through structural modeling, assessments of antimicrobial potency, and peptide-membrane calculations. The bacteriostatic and cytotoxic activities of the candidate peptides were

investigated, the results indicating that the modified peptide exhibited a low minimum inhibitory concentration for most bacteria and fungi [56]. Many potential compounds derived from crocodiles can be developed into new antibacterial drugs. The ongoing discovery and synthesis of novel antimicrobial peptides pave the way for practical applications in biological and medical fields, while also addressing issues associated with antibiotic overuse.

3.2. Anti-oxidant and Anti-aging

Oxidation reactions produce free radicals that can cause cell damage or death; antioxidants inhibit these reactions and play significant roles in food preservation and clinical applications [57]. Scientists have analyzed crocodile whole blood and isolated two short peptides that exhibited antioxidant activity [58]. Further experiments involving DPPH free radical scavenging, lipid peroxidation inhibition, and reducing capacity demonstrated that crocodile white blood cell extracts have a significant potential for developing antioxidant drugs [59]. These peptides were hydrolyzed using papain and tested for antioxidant activity, revealing a high reducing capacity [34]. These studies have significantly contributed to the clinical application of treatments for oxidative stress-related diseases.

Antioxidant properties are closely linked to anti-aging effects, as aging represents an imbalance between the damage caused by reactive oxygen species and the body's antioxidant defenses [60]. Crocodiles are long-lived reptiles [61]; therefore, researchers have explored whether the secrets of their longevity can be found in crocodile-derived substances. Li et al.,(2021) conducted a study on this issue [15]. They hydrolyzed crocodile meat using the Maillard reaction to obtain glycosylated peptides and tested their anti-aging effects using a fruit fly model. Although relevant studies on the anti-aging properties of crocodile peptides are limited and more in-depth research is needed, crocodile peptides hold great potential for anti-aging drug research.

3.3. Anti-tumor effect

Crocodiles live in harsh environments often contaminated with heavy metals, yet no cases of crocodile cancer have been reported, suggesting a strong immune system [62]. This makes crocodiles a promising subject for cancer research [63]. Based on this, numerous studies have identified around 100 peptides with potential anti-tumor properties, including the toxic effects of crocodile serum on cervical, breast, and prostate cancer cells [64].

The antitumor activity of crocodile white blood cell extract was evaluated in HeLa cells, and found that these extracts could induce HeLa cell apoptosis through both Caspase-dependent and independent pathways. Later, Maraming et al.,(2018) discovered the -RT2 peptide from crocodile white blood cells, which exhibited anti-tumor effects on human colon cancer xenotransplants in nude mice and induced apoptosis in human cervical cancer cells [65]. It was also extracted another active peptide, KT2, which could internalize through plasma membranes, alter cell morphology, and inhibit human melanoma cells by partially blocking the FAK pathway. Crocodile-derived

choline also showed a promise, reducing cyclin expression, arresting the cell cycle in the G2/M phase, and accelerating apoptosis via the mitochondrial pathway. It has shown a particular efficacy against human gastric cancer cells, suggesting a novel treatment approach [66]. Additionally, crocodile organ lysates demonstrated antitumor activity against prostate cancer cells, killing more than 60% of PC-3 cells [67]. These findings highlight the potential of crocodile peptides in cancer research. With cancer impacting countless families, developing anticancer drugs from these peptides could provide substantial societal benefits.

In a study by Siddiqui et al.,(2021), crocodile serum and heart lysate had up to a 70% mortality rate against prostate cancer cells. However, they did not exhibit any toxic effects on mammalian cells [63]. The KT2 and RT2 cationic antimicrobial peptides found in crocodiles were lethal to colon cancer HCT-116 cells, but the cell viability assay showed no toxic effects on non-cancerous Vero cells. Other studies also indicated that crocodile antimicrobial peptides show low cytotoxicity to macrophage cell lines. Collectively, these results indicate that crocodile peptides are weakly toxic to normal cells but toxic to cancer cells.

3.4. Anti-inflammatory

Inflammation is a physiological immune response caused by inflammatory factors that plays a restorative role during infection and injury [68]. Anti-inflammatory drugs are used to reduce inflammation or swelling and alleviate pain. Over time, many studies have examined the anti-inflammatory effects of crocodile peptides.

The basic mechanism of anti-inflammatory activity of crocodile hemoglobin was elucidated in many studies. When RAW264.7 cells were stimulated with LPS, crocodile hemoglobin treatment made nitric oxide (NO) production decrease, indicating that crocodile hemoglobin has anti-inflammatory activity [69]. Some scientists suggest that crocodiles' ability to survive in harsh environments may be influenced by their unique gut microbiota. Siddiqui et al.,(2023) isolated a strain from the intestinal tract of crocodiles, which showed a significant inhibitory effect on nitric oxide production, potentially offering a new therapeutic approach for intestinal diseases [70].

4. Perspectives

Crocodile peptides show significant potential with diverse effects. However, there are limited relevant studies, possibly because (1) artificial breeding of crocodiles is uncommon, and wild crocodiles are difficult to obtain, (2) studying crocodiles is costly, and (3) some crocodile species are endangered. More investigations are needed before clinical applications, including the effects on the human body, routes of administration, effective doses, and so on.

Active components of crocodile peptides have been extracted from white blood cells, organ lysates, hemoglobin, meat hydrolysates, and reprocessed peptides. Exploring the mechanisms of action and interaction with the human body can yield valuable insights. Current studies suggest that crocodile peptides have antibacterial

effects by damaging cell membranes, but the specific target remains unknown. Additionally, studies on RT2 have included animal experiments, but other peptides have only been studied at the cellular level, with no animal experiments.

Crocodile peptide products, such as functional food, dietary supplements, and drinks, are already marketed. However, the relative effectiveness of crocodile peptide products remains uncertain. Studies have demonstrated that crocodile peptides have antimicrobial, antioxidant, anticancer or anti-aging effects, but most of the studies are at the in vitro experimental stage, and further mechanistic studies are needed to confirm the specificity of their biological functions.

5. Conclusion

Crocodile peptides are widely distributed throughout crocodiles and exhibit antibacterial, antioxidant, anti-aging, anti-cancer, and anti-inflammatory properties, all while being non-toxic to normal mammalian cells. They are also abundant and can be sourced in large quantities, making them more accessible for extensive experimentation compared to other valuable biological samples.

Identifying the active components of crocodile peptides, along with conducting further animal testing, is vital for their development as therapeutic drugs. Given their promising biological activities, crocodile peptides represent a potentially effective solution for addressing various health issues. Continued research into their mechanisms of action, optimal dosages, and delivery methods is essential for translating these findings into clinical applications, paving the way for novel medical treatments.

6. Methodology

This article is based on studies published in English-language journals available in PubMed and Google Scholar as of December 2023. The following keywords were used to search for studies on crocodile peptides: "crocodile peptides," "antibacterial," "antioxidant," "anti-aging," "anti-tumor," "anti-cancer," and "anti-inflammatory," among others. Other relevant information was gathered according to the theme to fill the knowledge gap in crocodile peptide-related research.

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Conflict of Interest

The authors declare that they have no competing interests, and all authors confirm its accuracy.

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