Notice

DESCRIPTION CN120504718A

A method for synthesizing GHK-CU

[0001]

Technical Field

[n0001]

This invention belongs to the field of polypeptide synthesis technology, specifically relating to a method for synthesizing GHK-CU.

[0003]

Background Technology

[n0002]

GHK-Cu (blue copper peptide) is an active tripeptide composed of glycyl-histyl-lysine tripeptide bound to divalent copper ions. It is naturally found in human blood, saliva, and urine.

At age 20, the concentration of GHK in the blood is 200 ng/mL, but by age 60, due to a significant decline in the body's organ regeneration capacity, the concentration drops to 80 ng /mL.

In 1973, Pickart first isolated GHK-Cu from human plasma and discovered that copper peptides are very effective for wounds and skin injuries.

Founded in Washington, D.C. in 1986, ProCyte is dedicated to applying copper-bonded amino acids to the treatment of human tissue repair, anti-aging, wrinkle reduction, and hair growth. In 1999, the Neova brand was established, officially using blue copper for anti-aging and wrinkle removal beauty care. With a global exclusive patent, this groundbreaking GHK-Cu technology can increase skin vitality without harming or irritating the skin, and gradually restore the collagen lost in the body, making the subcutaneous tissue firm and strong, accelerating wound healing, and thus gradually achieving the purpose of wrinkle removal and anti-aging. GHK and its copper complex can act as activators of tissue remodeling. It is also a signaling peptide that can effectively promote the production of collagen and elastin, increase angiogenesis and antioxidant capacity, and stimulate the production of glycosaminoglycans (GAGs), helping the skin restore its self-repair ability. GHK-Cu is also an important factor in the human body that helps injured tissues fight inflammation, playing a vital role in stimulating the production of normal proteins to replace damaged proteins. Blue copper peptides have multiple functions, acting as both messengers and carriers. Secondly, it has advantages such as being gentle, non-irritating, and safe, thus it has high application value in topical skin care products.

[n0003]

Existing GHK-Cu synthesis technology suffers from several problems, including uneven copper ion dispersion due to traditional solvent systems, the formation of metal clusters that affect coordination reactions, poor thermodynamic control of reaction selectivity, a high proportion of byproducts, and local overheating caused by conventional heating methods that trigger amino acid racemization reactions. These issues severely limit the widespread application of blue copper peptides.

[n0004]

Based on this, we propose a synthesis method for GHK-CU, hoping to address the shortcomings of existing technologies.

[0007]

Summary of the Invention

[n0005]

The purpose of this invention is to address the existing problems by providing a method for synthesizing GHK-CU.

[n0006]

This invention is achieved through the following technical solution:

[0010]

A method for synthesizing GHK-CU includes the following steps:

[0011]

S1. Glycine, histidine, and lysine are dissolved in an ethanol-water mixture in a molar ratio of 1:1:1, and then subjected to CO₂expansion fluid treatment. After treatment for 30-40 minutes, crystals are precipitated and freeze-dried to obtain amino acid powder.

[0012]

S2. Prepare a 0.1M copper sulfate solution and a 0.2M reducing agent. Add the prepared copper sulfate solution and reducing agent to the microchannel reactor at the same time.

React at 30~50°C and a flow rate of 10mL/min. After the reaction is completed, collect the Cu (OH)₂@SiO₂ nanoparticle suspension.

[0013]

S3. 1-Butyl-3-methylimidazolium chloride and β-cyclodextrin modified with aspartic acid were melt-reacted at a molar ratio of 1:1~2 at 80~90°C to obtain a functionalized ionic liquid. Then, amino acid powder and Cu(OH)₂@SiO₂ nanoparticle suspension were added to the ionic liquid, stirred and mixed, and the pH was adjusted to 5~6. The mixture was then added to a microwave-ultrasonic composite reactor for reaction.

[0014]

S4. After the reaction is complete, cool to room temperature, add acetone to precipitate the ionic liquid, centrifuge to collect the supernatant, filter the supernatant and then pass it through reverse C18 column chromatography to collect the elution peak of the copper peptide, and freeze-dry.

[n0007]

Furthermore, in step S1, the volume ratio of ethanol to water in the ethanol-water mixed solvent is 1:4~5;

[0016]

When processing CO₂ expansion fluid, the pressure is set to 15~20MPa, the temperature to 40~45°C, and the flow rate to 400~500mL/min.

[n0008]

Furthermore, the reducing agent mentioned in step S2 is ascorbic acid, and the reducing agent contains tetraethyl orthosilicate, with a molar ratio of tetraethyl orthosilicate to Cu²⁺ in the reaction system of 2~3:1;

[0018]

The inner diameter of the microchannel reactor is 500 μm.

[n0009]

Furthermore, the preparation of the aspartic acid-modified β-cyclodextrin described in step S3 includes the following steps:

[0020]

(1) Dissolve β-cyclodextrin in dimethyl sulfoxide, add sodium hydroxide and stir well, then add propargyl bromide dropwise and react at room temperature for 20-26 h. After the

reaction is complete, pour into ice water to precipitate the precipitate, filter, wash with deionized water 4-6 times, and dry to obtain pretreated β-cyclodextrin.

[0021]

(2) Dissolve polyaspartic acid in dichloromethane, add N,N-dicyclohexylcarbodiimide at 1.3 to 1.4 times the molar amount of polyaspartic acid, stir and mix well, then add sodium azide at 1 to 2 times the molar amount of polyaspartic acid, react at room temperature for 16 to 18 hours, filter after the reaction, concentrate the filtrate, precipitate with diethyl ether, filter and dry to obtain pretreated polyaspartic acid;

[0022]

(3) Add pretreated β-cyclodextrin and aspartic acid to phosphate buffer at a molar ratio of 1: 1~2, adjust the pH to 6~8, add transglutaminase, stir and react at 30~40°C for 8~10h, add pretreated aspartic acid, stir and mix well, add copper sulfate and sodium ascorbate, stir and react at room temperature for 16~20h, the reaction ends, remove unreacted raw materials by chromatography to obtain aspartic acid modified β-cyclodextrin.

[n0010]

Further, the molar ratio of β-cyclodextrin to propargyl bromide in step (1) is 1:1~3;

[0024]

The amount of sodium hydroxide added is 0.6~1% of the mass of β-cyclodextrin.

[n0011]

Furthermore, the amount of transglutaminase added in step (3) is 3-4% of the total mass of β-cyclodextrin and aspartic acid;

[0026]

The molar ratio of pretreated β-cyclodextrin to pretreated aspartic acid was 1:1.3~1.7;

[0027]

The concentration of copper sulfate is 0.03~0.07mM, and the concentration of sodium ascorbate is 0.3~0.7mM.

[n0012]

Furthermore, in step S3, when adding the amino acid powder and Cu(OH)₂@SiO₂ nanoparticle suspension to the ionic liquid, the total concentration of amino acids is controlled to be 0.5~1M, and the molar ratio of Cu²⁺ to amino acids is 1:1.2~1.4.

[n0013]

Furthermore, when adding the material to the microwave-ultrasonic composite reactor for reaction as described in step S3, the microwave parameters are as follows: dual-frequency alternating irradiation is used, with 2.45 GHz and 5.8 GHz cycling for 10 seconds each, the initial power density is 50 W/cm_NER12, and it increases by 50 W/cm_NER13 to 200 W /cm_NER14 every 5 minutes.

[0030]

Ultrasonic parameters: frequency 20~100kHz, using a pulse mode of 30s working and 10s pausing.

[n0014]

Furthermore, the supernatant filtration in step S4 is performed using an ultrafiltration membrane with a pore size of 10 kDa.

[0032]

The mobile phase for reverse C18 column chromatography was acetonitrile and water in a 2:8 ratio, containing 0.1% trifluoroacetic acid.

[n0015]

The present invention has the following advantages over the prior art:

[0034]

1. This invention provides a method for synthesizing GHK-CU (blue copper peptide). By pretreating the raw materials and improving the reaction system, the selectivity and efficiency

of the reaction are significantly improved. During the reaction, microwave and ultrasonic means are used to promote the reaction and avoid local overheating or side reactions, thereby improving the yield and purity of the product. The synthesized blue copper peptide has excellent firming, anti-wrinkle and wound healing effects.

[n0016]

2. This invention uses CO₂expansion fluid technology to recrystallize glycine, histidine, and lysine into small-diameter crystals with D90 < 10 µm, thereby increasing their specific surface area and improving their dissolution rate and reactivity.

Copper sulfate reacts with a reducing agent in a microchannel reactor to generate 20-50 nm Cu(OH)2@SiO₂ core-shell nanoparticles. The SiO₂ shell can control the slow release rate of Cu²⁺, avoid agglomeration, and improve the activity and uniform dispersion of copper ions in subsequent reactions, thereby improving the reaction efficiency.

[n0017]

3. This invention adds a specially formulated functionalized ionic liquid to the reaction system. The cation of the ionic liquid (1-butyl-3-methylimidazolium) provides a polar environment, while the anion (β-cyclodextrin modified with aspartic acid) has three functions. The cavity of the β-cyclodextrin modified with aspartic acid recognizes and includes amino acids, orienteding their functional groups. The carboxyl group of aspartic acid is pre-coordinated with Cu²⁺ to form a copper ion-carboxyl intermediate, reducing the activation energy of the coordination reaction. The β-cyclodextrin modified with aspartic acid can form a complex with copper peptide, increasing the solubility of copper peptide in the reaction system, allowing it to be better dispersed in the solution and fully contact other reactants, which is beneficial to the reaction.

[n0018]

4. This invention combines microwave and ultrasound. Microwave dual-frequency irradiation (alternating between 2.45GHz and 5.8GHz) generates a high-frequency alternating electromagnetic field, which accelerates molecular motion and promotes the collision and coordination of amino acids and copper ions. Power density gradient control (50~300W /cm²) achieves temperature gradient regulation. At the same time, with the help of ultrasound, the outer shell of nanoparticles is broken, releasing Cu²⁺, further improving the efficiency of the reaction.

[0038]

Attached Figure Description

[n0019]

Figure 1 shows the phenotypic diagram of the efficacy experiment of 0.5 ppm blue copper peptide;

[0040]

Figure 2 shows the phenotypic diagram of the efficacy experiment of 20ppm blue copper peptide;

[0041]

Figure 3 shows the comparison of elastase inhibition rates between the sample group and the blank group.

[0042]

Detailed Implementation

[n0020]

To further explain the present invention, the following specific embodiments are described.

[n0021]

Example 1

[0045]

A method for synthesizing GHK-CU includes the following steps:

[0046]

S1. Glycine, histidine, and lysine were dissolved in an ethanol-water mixed solvent (ethanol to water volume ratio of 1:4) at a molar ratio of 1:1:1. Then, CO₂ expansion fluid treatment was performed with a pressure of 15 MPa, a temperature of 40 °C, and a CO₂ flow rate of 400 mL/min. After treatment for 30 min, crystals precipitated and were freeze-dried to obtain amino acid powder.

[0047]

S2. Prepare a 0.1M copper sulfate solution and a 0.2M reducing agent. Add the prepared copper sulfate solution and reducing agent simultaneously into a microchannel reactor (with an inner diameter of 500μm). React at 30°C and a flow rate of 10mL/min. After the reaction is complete, collect the Cu(OH)₂@SiO₂ nanoparticle suspension.

[0048]

The reducing agent is ascorbic acid, and the reducing agent contains tetraethyl orthosilicate. The molar ratio of tetraethyl orthosilicate to Cu²⁺ in the reaction system is 2:1.

[0049]

S3. 1-Butyl-3-methylimidazolium chloride and aspartic acid-modified β-cyclodextrin were melt-reacted at 80°C in a 1:1 molar ratio to obtain a functionalized ionic liquid. Then, amino acid powder and Cu(OH)₂@SiO₂ nanoparticle suspension were added to the ionic liquid, and the total concentration of amino acids was controlled at 0.5M, and the molar ratio of Cu²⁺ to amino acids was 1:1.2. After stirring and mixing, the pH was adjusted to 5, and then the mixture was added to a microwave-ultrasonic composite reactor for reaction.

[0050]

Microwave parameters: Dual-frequency alternating irradiation was used, with 2.45 GHz and 5.8 GHz cycling for 10 seconds each. The initial power density was 50 W/cm_NER30, which was increased by 50 W/cm_NER31 to 200 W/cm_NER32 every 5 minutes.

[0051]

Ultrasound parameters: frequency 20kHz, using a pulse mode of 30s operation followed by 10s pause;

[0052]

The preparation of the aspartic acid-modified β-cyclodextrin includes the following steps:

[0053]

(1) Dissolve β-cyclodextrin in dimethyl sulfoxide, add sodium hydroxide and stir well, then add propargyl bromide dropwise and react at room temperature for 20 h. After the reaction is completed, pour into ice water to precipitate the precipitate, filter, wash with deionized water 4 times and dry to obtain pretreated β-cyclodextrin.

[0054]

The molar ratio of β-cyclodextrin to propargyl bromide is 1:1;

[0055]

The amount of sodium hydroxide added is 0.6% of the mass of β-cyclodextrin;

[0056]

(2) Dissolve polyaspartic acid in dichloromethane, add N,N-dicyclohexylcarbodiimide with a molar amount of 1.3 times that of polyaspartic acid, stir and mix well, then add sodium azide with a molar amount of 1 times that of polyaspartic acid, react at room temperature for 16 h, filter after the reaction is complete, concentrate the filtrate, precipitate with diethyl ether, filter and dry to obtain pretreated polyaspartic acid;

[0057]

(3) Add pretreated β-cyclodextrin and aspartic acid to phosphate buffer at a molar ratio of 1: 1, adjust the pH to 6, add transglutaminase, stir and react at 30°C for 8 hours, add pretreated aspartic acid, stir and mix well, add copper sulfate and sodium ascorbate, stir and react at room temperature for 16 hours, the reaction is completed, remove unreacted raw materials by chromatography to obtain aspartic acid modified β-cyclodextrin;

[0058]

The amount of transglutaminase added is 3% of the total mass of β-cyclodextrin and aspartic acid;

[0059]

The molar ratio of pretreated β-cyclodextrin to pretreated aspartic acid was 1:1.3;

[0060]

The concentration of copper sulfate is 0.03 mM, and the concentration of sodium ascorbate is 0.3 mM;

[0061]

S4. After the reaction is complete, cool to room temperature, add acetone to precipitate the ionic liquid, centrifuge to collect the supernatant, filter the supernatant through a 10 kDa ultrafiltration membrane and then pass it through reverse C18 column chromatography (mobile phase is acetonitrile and water in a 2:8 ratio, containing 0.1% trifluoroacetic acid), collect the blue copper peptide elution peak, and freeze-dry.

[n0022]

Example 2

[0063]

A method for synthesizing GHK-CU includes the following steps:

[0064]

S1. Glycine, histidine, and lysine were dissolved in an ethanol-water mixed solvent (ethanol to water volume ratio of 1:4.5) at a molar ratio of 1:1:1. Then, CO₂ expansion fluid treatment was performed at a pressure of 17MPa, a temperature of 43°C, and a CO₂ flow rate of 450mL/min. After treatment for 35min, crystals precipitated and were freeze-dried to obtain amino acid powder.

[0065]

S2. Prepare a 0.1M copper sulfate solution and a 0.2M reducing agent. Add the prepared copper sulfate solution and reducing agent simultaneously into a microchannel reactor (with an inner diameter of 500μm). React at 40°C and a flow rate of 10mL/min. After the reaction is complete, collect the Cu(OH)₂@SiO₂ nanoparticle suspension.

[0066]

The reducing agent is ascorbic acid, and the reducing agent contains tetraethyl orthosilicate. The molar ratio of tetraethyl orthosilicate to Cu²⁺ in the reaction system is 2.5:1.

[0067]

S3. 1-Butyl-3-methylimidazolium chloride and aspartic acid-modified β-cyclodextrin were melt-reacted at 85°C in a molar ratio of 1:1.5 to obtain a functionalized ionic liquid. Then, amino acid powder and Cu(OH)₂@SiO₂ nanoparticle suspension were added to the ionic liquid, and the total concentration of amino acids was controlled at 0.7M, and the molar ratio of Cu²⁺ to amino acids was 1:1.3. After stirring and mixing, the pH was adjusted to 5.5, and then the mixture was added to a microwave-ultrasonic composite reactor for reaction.

[0068]

Microwave parameters: Dual-frequency alternating irradiation was used, with 2.45 GHz and 5.8 GHz cycling for 10 seconds each. The initial power density was 50 W/cm², which was increased by 50 W/cm² to 200 W/cm² every 5 minutes.

[0069]

Ultrasonic parameters: frequency 60kHz, using a pulse mode of 30s operation followed by 10s pause;

[0070]

The preparation of the aspartic acid-modified β-cyclodextrin includes the following steps:

[0071]

(1) Dissolve β-cyclodextrin in dimethyl sulfoxide, add sodium hydroxide and stir well, then add propargyl bromide dropwise and react at room temperature for 23 h. After the reaction is completed, pour into ice water to precipitate the precipitate, filter, wash with deionized water 5 times and dry to obtain pretreated β-cyclodextrin.

[0072]

The molar ratio of β-cyclodextrin to propargyl bromide is 1:2;

[0073]

The amount of sodium hydroxide added is 0.8% of the mass of β-cyclodextrin;

[0074]

(2) Dissolve polyaspartic acid in dichloromethane, add N,N-dicyclohexylcarbodiimide at 1.35 times the molar amount of polyaspartic acid, stir and mix well, then add sodium azide at 1.5 times the molar amount of polyaspartic acid, react at room temperature for 17 h, filter after the reaction, concentrate the filtrate, precipitate with diethyl ether, filter and dry to obtain pretreated polyaspartic acid;

[0075]

(3) Add pretreated β-cyclodextrin and aspartic acid to phosphate buffer at a molar ratio of 1: 1.5, adjust the pH to 7, add transglutaminase, stir and react at 35°C for 9 h, add pretreated aspartic acid, stir and mix well, add copper sulfate and sodium ascorbate, stir and react at room temperature for 18 h, the reaction ends, remove unreacted raw materials by chromatography to obtain aspartic acid modified β-cyclodextrin;

[0076]

The amount of transglutaminase added is 3.5% of the total mass of β-cyclodextrin and aspartic acid;

[0077]

The molar ratio of pretreated β-cyclodextrin to pretreated aspartic acid was 1:1.5;

[0078]

The concentration of copper sulfate is 0.05 mM, and the concentration of sodium ascorbate is 0.5 mM;

[0079]

S4. After the reaction is complete, cool to room temperature, add acetone to precipitate the ionic liquid, centrifuge to collect the supernatant, filter the supernatant through a 10 kDa ultrafiltration membrane and then pass it through reverse C18 column chromatography (mobile phase is acetonitrile and water in a 2:8 ratio, containing 0.1% trifluoroacetic acid), collect the blue copper peptide elution peak, and freeze-dry.

[n0023]

Example 3

[0081]

A method for synthesizing GHK-CU includes the following steps:

[0082]

S1. Glycine, histidine, and lysine were dissolved in an ethanol-water mixed solvent (ethanol to water volume ratio of 1:5) at a molar ratio of 1:1:1. Then, CO₂ expansion fluid treatment was performed at a pressure of 20 MPa, a temperature of 45 °C, and a CO₂ flow rate of 500 mL/min. After treatment for 40 min, crystals precipitated and were freeze-dried to obtain amino acid powder.

[0083]

S2. Prepare a 0.1M copper sulfate solution and a 0.2M reducing agent. Add the prepared copper sulfate solution and reducing agent simultaneously into a microchannel reactor (with an inner diameter of 500μm). React at 50°C and a flow rate of 10mL/min. After the reaction is complete, collect the Cu(OH)₂@SiO₂ nanoparticle suspension.

[0084]

The reducing agent is ascorbic acid, and the reducing agent contains tetraethyl orthosilicate. The molar ratio of tetraethyl orthosilicate to Cu²⁺ in the reaction system is 3:1.

[0085]

S3. 1-Butyl-3-methylimidazolium chloride and aspartic acid-modified β-cyclodextrin were melt-reacted at 90°C in a molar ratio of 1:2 to obtain a functionalized ionic liquid. Then, amino acid powder and Cu(OH)₂@SiO₂ nanoparticle suspension were added to the ionic liquid, and the total concentration of amino acids was controlled at 1M. The molar ratio of Cu²⁺ to amino acids was 1:1.4. After stirring and mixing, the pH was adjusted to 6, and then the mixture was added to a microwave-ultrasonic composite reactor for reaction.

[0086]

Microwave parameters: Dual-frequency alternating irradiation was used, with 2.45 GHz and 5.8 GHz cycling for 10 seconds each. The initial power density was 50 W/cm², which increased by 50 W/cm² to 200 W/cm² every 5 minutes.

[0087]

Ultrasonic parameters: frequency 100kHz, using a pulse mode of 30s operation followed by 10s pause;

[0088]

The preparation of the aspartic acid-modified β-cyclodextrin includes the following steps:

[0089]

(1) Dissolve β-cyclodextrin in dimethyl sulfoxide, add sodium hydroxide and stir well, then add propargyl bromide dropwise and react at room temperature for 26 h. After the reaction is completed, pour into ice water to precipitate the precipitate, filter, wash with deionized water 6 times and dry to obtain pretreated β-cyclodextrin.

[0090]

The molar ratio of β-cyclodextrin to propargyl bromide is 1:3;

[0091]

The amount of sodium hydroxide added is 1% of the mass of β-cyclodextrin;

[0092]

(2) Dissolve polyaspartic acid in dichloromethane, add N,N-dicyclohexylcarbodiimide with a molar amount of 1.4 times that of polyaspartic acid, stir and mix well, then add sodium azide with a molar amount of 2 times that of polyaspartic acid, react at room temperature for 18 hours, filter after the reaction, concentrate the filtrate, precipitate with diethyl ether, filter and dry to obtain pretreated polyaspartic acid;

[0093]

(3) Add pretreated β-cyclodextrin and aspartic acid to phosphate buffer at a molar ratio of 1: 2, adjust the pH to 8, add transglutaminase, stir and react at 40°C for 10 h, add pretreated aspartic acid, stir and mix well, add copper sulfate and sodium ascorbate, stir and react at room temperature for 20 h, and after the reaction is completed, remove unreacted raw materials by chromatography to obtain aspartic acid modified β-cyclodextrin;

[0094]

The amount of transglutaminase added is 4% of the total mass of β-cyclodextrin and aspartic acid;

[0095]

The molar ratio of pretreated β-cyclodextrin to pretreated aspartic acid was 1:1.7;

[0096]

The concentration of copper sulfate is 0.07 mM, and the concentration of sodium ascorbate is 0.7 mM;

[0097]

S4. After the reaction is complete, cool to room temperature, add acetone to precipitate the ionic liquid, centrifuge to collect the supernatant, filter the supernatant through a 10 kDa ultrafiltration membrane and then pass it through reverse C18 column chromatography (mobile phase is acetonitrile and water in a 2:8 ratio, containing 0.1% trifluoroacetic acid), collect the blue copper peptide elution peak, and freeze-dry.

[n0024]

Comparative Example 1

[0099]

Based on Example 2, in step S1, CO₂expansion fluid treatment is not performed. Instead, glycine, histidine, and lysine are freeze-dried directly in a molar ratio of 1:1:1. The remaining technical solutions are consistent with those in Example 2.

[n0025]

Comparative Example 2

[0101]

Based on Example 2, the aspartic acid-modified β-cyclodextrin in step S3 was replaced with untreated β-cyclodextrin, and the rest of the technical solutions were the same as those in Example 2.

[n0026]

Comparative Example 3

[0103]

Based on Example 2, the step S3 of adding the material to the microwave-ultrasonic composite reactor for reaction is omitted and replaced with direct stirring. The remaining technical solutions are the same as those in Example 2.

[n0027]

1. Yield and purity tests

[0105]

Blue copper peptides were prepared using the methods described in Examples 1-3 and Comparative Examples 1-3, respectively, and then characterized by HPLC.

The experimental results are shown in Table 1 below.

[n0028]

Table 1

[n0029]

As shown in Table 1 above, the present invention significantly improves yield and purity and shortens reaction time through raw material activation, directional coordination of ionic liquids, and energy field synergy.

[n0030]

2. Blue copper peptide zebrafish maintenance effects

[0110]

Experimental system: Wild-type AB strain zebrafish.

[n0031]

Zebrafish age: 3 days after fertilization (3dpf).

[n0032]

Sample size per group: 15 tails (N=10).

[n0033]

Adult fish rearing and breeding methods: The rearing and breeding methods shall be carried out in accordance with the applicant's laboratory standards and meet the requirements of international AAALAC certification (certification number: 001458).

[n0034]

Testing agency: Guangzhou Huante Zhiyu Youjian Biotechnology Co., Ltd.

[n0035]

Zebrafish tail fin regeneration consists of three processes: wound healing, bud formation, and regeneration outcome, with bud formation being the most crucial.

The cells that promote fin regeneration come from multiple sources, including various cell types such as epidermal cells, fibroblasts, and osteoblasts, and these cells are highly lineage-restricted.

The regeneration mechanism of zebrafish tail fins is similar to that of human skin, bones, and blood vessels.

The zebrafish tail fin has become an important model for studying tissue regeneration processes due to its simple structure, ease of surgical manipulation, lack of impact on postoperative survival, and ease of observation.

The repair efficacy of zebrafish samples was evaluated by quantifying the area of the regenerated caudal fin after the caudal fin was severed perpendicularly to the body using a scalpel.

[n0036]

Experimental methods

[0117]

(1) A zebrafish tail fin injury model was established by surgically removing the zebrafish tail fin;

[0118]

(2) Randomly distribute the model zebrafish into 6-well plates, with 15 fish per well;

[0119]

(3) Water-soluble samples were administered (Example 2), and a normal control group and a model control group were set up at the same time, with a volume of 3 mL per well;

[0120]

(4) Incubate at 28°C in the dark for 48 hours;

[0121]

(5) Ten zebrafish were randomly selected from each experimental group and photographed under a dissecting microscope. Advanced image processing software was used to analyze and collect data. The area of the zebrafish tail fin (A) was analyzed, and the formula was used to calculate and determine whether the sample had a repair effect.

[n0038]

Statistical analysis: $P < 0.05$, which was considered to be a significant difference.

[n0039]

The test results are shown in Table 2 and Figures 1 and 2.

[n0040]

Table 2

[n0041]

As shown in Table 1 and Figures 1 and 2, the tail fin area of the 0.5 ppm and 20 ppm copper peptide samples was significantly increased compared with that of the model control group, revealing that the sample has a repairing effect.

[n0042]

2. Anti-wrinkle and firming effects (elastase inhibition rate test)

[0129]

Experimental methods

[0130]

(1) Add 0.2 mL of sample solution of the same concentration to the sample tube and the sample background (Example 2), add 1.0 mL of pH 8.8 borate buffer to the sample

background, add 0.2 mL of pH 8.8 borate buffer to the enzyme reaction tube, and add 1.0 mL of pH 8.8 borate buffer to the solvent background.

[n0043]

(2) Add 10 mg of lichen red-elastin and 1.0 mL of elastase to the sample tube and enzyme reaction tube, respectively.

Mix the mixture thoroughly and place it in a constant temperature water bath shaker for 20 minutes.

[n0044]

(3) Add 2 mL of pH 6.0 phosphate buffer to each sample tube, sample background, enzyme reaction tube and solvent background, and then use an equal volume of pH 8.8 borate buffer and pH 6.0 phosphate buffer to make up to 5 mL; then centrifuge for 5 min and take 3 mL of the supernatant.

[n0045]

(4) Transfer each reaction solution into a 1cm cuvette and measure the absorbance at 590nm.

[n0046]

The experimental results are shown in Table 3 and Figure 3.

[n0047]

Table 3

[n0048]

Note: For the significance analysis of the sample group and the blank group, $P < 0.05$ was considered statistically significant. The P value of the comparison between the two groups was 0.000, indicating a significant difference.

[n0049]

As shown in Table 2 and Figure 3, under the experimental conditions, the elastase inhibition rate of the sample tripeptide-1 copper was detected. When the sample concentration was 0.1%, 0.2%, and 0.3%, the elastase inhibition rates were 6.95%, 11.47%, and 22.78%, respectively. Compared with the blank group, the elastase inhibition rates of the sample group at each concentration were better than those of the blank group, and the differences were significant ($P < 0.05$).

This indicates that tripeptide-1 copper has an inhibitory effect on elastase under the experimental conditions, and has certain anti-wrinkle and firming effects.

[n0050]

The above description is only a preferred embodiment of the present invention, but the scope of protection of the present invention is not limited thereto. Any equivalent substitutions or modifications made by those skilled in the art within the scope of the technology disclosed in the present invention, based on the technical solution and inventive concept of the present invention, should be covered within the scope of protection of the present invention.
