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Research Article

EVALUATION OF THE ANTI-AGING AND WHITENING EFFICACY OF COLLAGEN TRIPEPTIDE AND CHICKEN PROTEIN HYDROLYSATE

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ABSTRACT. Aging is one of the inevitable problems people face, age can cause significant change in skin, both inside and out. As such, ingredients that can delay the aging of skin are highly valued. There are many peptides that have been found to be beneficial to human health, they can reduce the risk of diseases and can be used as anti-aging ingredients. Previous studies have evaluated collagen tripeptide and chicken protein hydrolysate. The studies reported that collagen tripeptide and chicken protein hydrolysate can enhance wound healing and improve physical stamina, respectively. Collagen tripeptide and chicken protein hydrolysate have the potential for applications in a variety of fields. In this study, reconstructed human epidermal were used *in vitro* skin irritation tests to test anti-aging and whitening effects, and the tests were used to evaluate the safety and efficacy of collagen tripeptide and chicken protein hydrolysate. The results showed that collagen tripeptide and chicken protein hydrolysate were non-irritant. At 20 mg/mL, the collagen type I synthesis of chicken protein hydrolysate and collagen tripeptide were increased to 99.3% and 129.4% and promoted fibroblast proliferation to 62.2% and 22.1%. 4 mg/mL of chicken protein hydrolysate can inhibit melanin production at 29.7% (with α-MSH stimulation). There is the potential for use in cosmetics, and may be a potential candidate for development in anti-aging and whitening.

Keywords: *Peptides, skin irritation, anti-aging, whitening.*

INTRODUCTION

The aging of skin in humans is a complicated process, and skin aging is related to changes in skin structure and function [1]. The elasticity of human skin is mainly supported by collagen in the dermis [2]. Wrinkles are caused by collagen matrix degradation [3]. Without strengthening the production of collagen, the skin will lack support, resulting in lack of elasticity, wrinkles, skin frailty, and dullness [4, 5]. At present, many new peptides have been developed. Some peptides may have the potential to benefit human health, reducing the risk of disease and using it as an anti-aging ingredient [6]. Peptides are developed as raw materials and used in the fields of food, nutrition, and cosmetics [7, 8, 9]. Peptides are amino acid chains that are made up of 2 to

20 units and has a molecular weight lower than 6000 Da [10]. Unlike large proteins, peptides can penetrate the skin more easily and reach deeper skin layers [11].

In previous studies, collagen tripeptide and chicken protein hydrolysate have emphasised that collagen tripeptide has the potential as an anti-photoaging agent, and might be a beneficial choice for encourage the healing of skin wounds and promote recovery after laser treatment of skin [12, 13]. Chicken protein hydrolysate, on the other hand, has the potential to improve physical stamina, alleviating fatigue, and lower serum cholesterol [14, 15].

In this study, an *in vitro* skin irritation test using reconstructed human epidermal and a Thiazolyl Blue Tetrazolium Bromide (MTT) assay were used to evaluate the safety effect of collagen tripeptide and chicken protein hydrolysate. The anti-aging effect and whitening effect were measured to evaluate the possibility of collagen tripeptide and chicken protein hydrolysate as cosmetic raw materials as well as other applications.

MATERIALS AND METHODS

Chemicals and reagents

Arbutin, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), α-melanocyte stimulating hormone (a-MSH), Bradford and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), trypsin/EDTA and phosphate buffered saline (PBS) were purchased from Corning (Corning, NY, USA). Transforming Growth Factor-β1 human (TGF-β) was purchased from R&D Systems (Minneapolis, MN). Procollagen Type I C-Peptide (PIP) EIA Kit was purchased from TAKARA BIO INC (Shiga, Japan).

Sample preparation

Collagen tripeptide and chicken protein hydrolysate were supplied by JELLICE PIONEER PRIVATE LIMITED TAIWAN BRANCH (SINGAPORE). According to the manufacturer, collagen tripeptide was obtained by the enzymatic degradation of the skin of Sutchi catfish (*Pangasius hypophthalmus*) [12]. Chicken protein hydrolysate was hydrolyzed enzymatically from fresh chicken breast meat at pH 8.5, 55 ℃ for 5 h, by Protamex™ (Novo Nordisk A/S Co., Bagsvaerd, Denmark) [15].

Cell culture

Human foreskin fibroblast Hs68 cells and B16F10 mouse melanoma cells were obtained from the Cell Culture Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Hs68 cells and B16F10 cells were cultured in DMEM with 10% FBS and 1% P/S. The cells were cultured at 37°C in a humidified incubator with 5% CO_2.

Skin irritation test

The EpiDerm™ (Reconstructed Human Epidermal Model) was used in the *in vitro* Skin Irritation Test. The tissue was obtained and preserved in accordance to the manual by MatTek (Ashland, MA, USA). The *in vitro* skin irritation test followed the Organization for Economic Cooperation and Development Test Guideline 439 (OECD

TG 439). The tissues were exposed to the test substances in triplicate. Then the different test substance and relative tissue viability were compared. As stated in the acceptance criteria, if the tissue viability of the test substance is below 50% of the viability of the negative control, it should be classified as irritating [16].

Procollagen type I synthesis assay

Hs68 cells were seeded into 96-well plates (8×10^3 cells/well) and cultured overnight. Serum-free medium containing drugs were refreshed in each well and incubated for 72 hr. The supernatants were harvested and the procollagen type I C-peptide (PIP) was measured with a procollagen type I C-peptide ELISA kit (Takara, Otsu, Japan) according to the manufacturer's instructions.

MTT assay

The MTT assay coupled with the PIP assay was used to measure cell viability and cell proliferation. After the supernatant were harvested, 100 μL of 1 mg/Ml MTT solution was added to each well for 2 hr. After that, the MTT was removed and 100 μL of DMSO was added to each well [17]. A plate reader was used to determine the absorbance at 570 nm.

Melanin content assay

B16F10 cells were seeded into 12-well plates (4×10^4 cells/well) and cultured overnight. Serum-free medium containing drugs were refreshed in each well and incubated for 48 hr with or without α-MSH (30 nM). At the end of the treatments, the cells were washed with PBS and lysed with 350 μL of NaOH for 1 hr at 55 °C. Absorbance at 405 nm was determined with a plate reader.

Bradford assay

A part of the cells lysed by NaOH was used for the melanin content assay, and the remaining cells were used to measure the cell viability. 200 μL of Bradford reagent was added to each well and shook for 5 min. Absorbance at 595 nm was determined with a plate reader.

Statistics analysis

The data were all shown by the mean with the standard deviation (mean ±SD). Student t-test was used to examine significant differences between results. The *p*-value of less than 0.05 was statistically significant.

RESULTS AND DISCUSSION

Skin irritation test

In vitro skin irritation test was based on OECD TG 439, a non-irritant is determined if the mean tissue viability of test samples were greater than 50% of those of the negative controls. PBS was used as the negative control and 5% sodium dodecyl sulfate (SDS) was used as the positive control. The relative viability of chicken protein hydrolysate and collagen tripeptide were 94.7% and 100.8%, respectively (Fig. 1). According to this

result, chicken protein hydrolysate and collagen tripeptide can be classified as non-irritant.

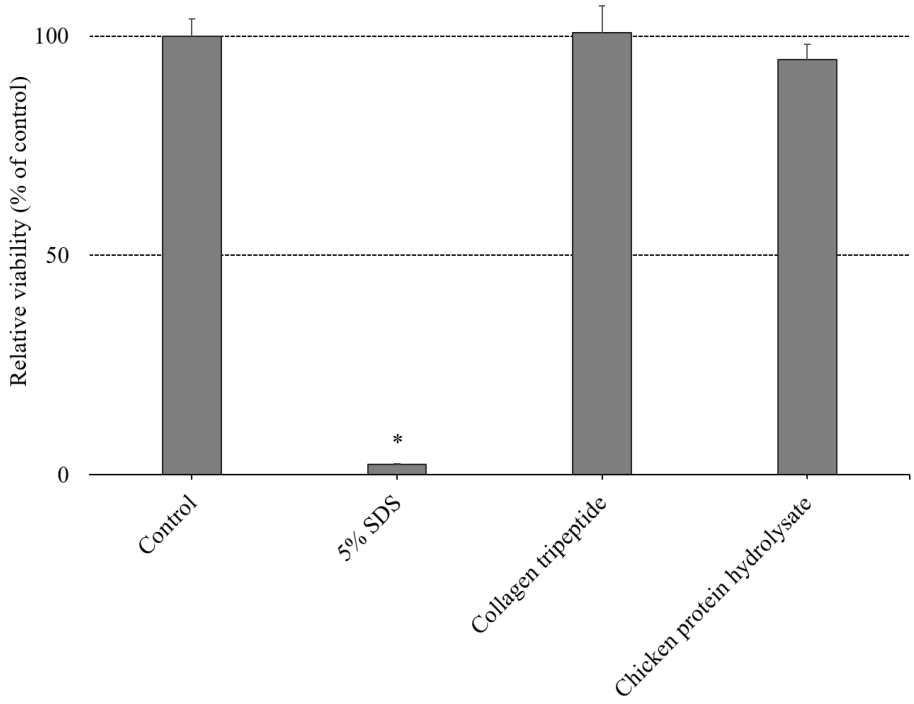

Fig. 1. *In vitro skin irritation test. Negative control: PBS, positive control: 5% SDS, and test substance: the powder of chicken protein hydrolysate and collagen tripeptite. *p<0.05 compared to control.*

Measurement of procollagen Type I C-Peptide

Collagen type I, II, III, IV and V are procollagens, which are precursor molecules [18]. Among them, collagen type I constitutes approximately 85% of total collagens [19]. Collagen type I is most commonly used in product manufacturing in a variety of cosmetic applications due to its high biocompatibility [20], making it an excellent target in the anti-aging process. Therefore, this study evaluates how much type I collagens were synthesized. In skin fibroblasts, a major activator of collagen synthesis is TGF-β [19]. Thus, the positive control was 10 ng/mL of TGF-β [21] and the expression of collagen type I was increased by 48.4% (Fig. 2 and 3). Chicken protein hydrolysate showed a significant increase in collagen type I synthesis by 28.2%, 46.7%, 80.4% and 99.3% at the concentration of 2.5, 5, 10 and 20 mg/mL, respectively (Fig. 2). At the concentration of 2.5, 5, 10 and 20 mg/mL of collagen tripeptide, a significant increase in collagen type I synthesis was at 36.7%, 58.8%, 86.9% and 129.4%, respectively (Fig. 3).

Wrinkles form as the collagen matrix degenerates. In order to regenerate the matrix and produce more collagens, the stimulation of fibroblasts is needed [22]. Cell viability was confirmed using the MTT assay and the ability of test substances in promoting the proliferation of fibroblasts was evaluated. Figure 2 and 3 showed that the proliferation of fibroblasts treated with TGF-β was 23.7% (10 ng/mL). The proliferation of fibroblasts treated with chicken protein hydrolysate was 23.8%, 32.3%, 52.1% and 62.2% at concentrations of 2.5-20 mg/mL (Fig. 2). The proliferation of fibroblasts treated with

collagen tripeptide was 11.8%, 16.7%, 10.5% and 22.1% at concentrations of 2.5-20 mg/mL (Fig. 3). Chicken protein hydrolysate and collagen tripeptide have the ability to increase collagen type I synthesis and promote the proliferation of fibroblasts.

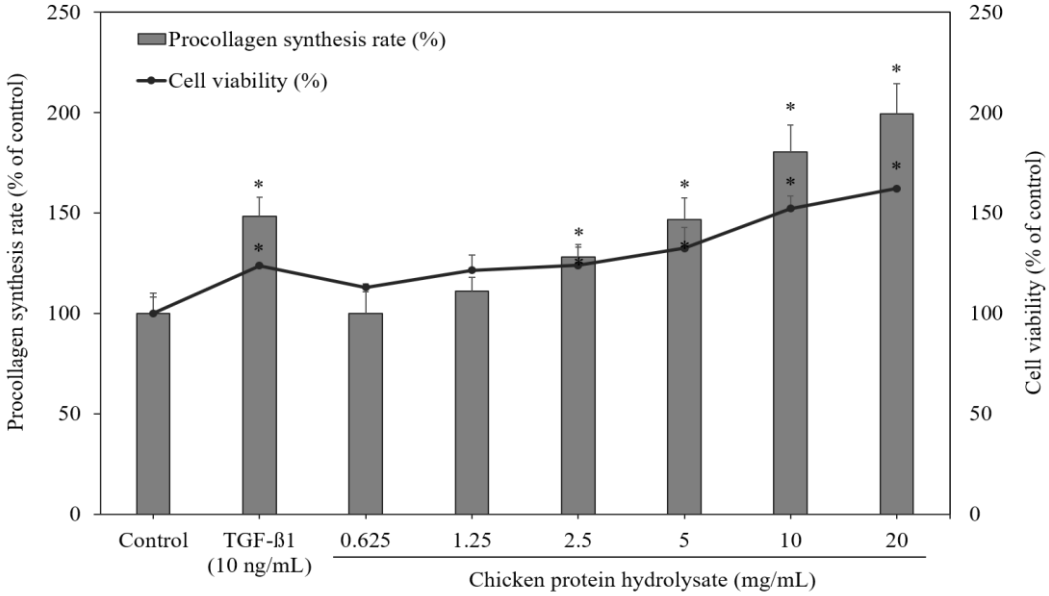

Fig. 2. *Collagen Type I synthesis of chicken protein hydrolysate on Hs68. Negative control: DI water, positive control: TGF-β1 (10 ng/mL), and test substance: chicken protein hydrolysate. *p<0.05 compared to control.*

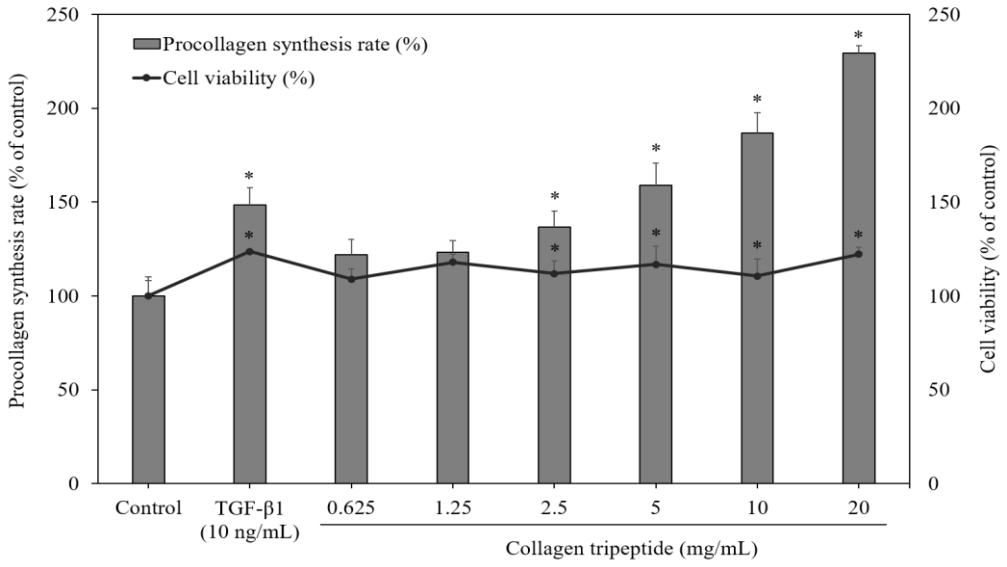

Fig. 3. *Collagen Type I synthesis of collagen tripeptide on Hs68. Negative control: DI water, positive control: TGF-β1 (10 ng/mL), and test substance: collagen tripeptide. *p<0.05 compared to control.*

Measurement of cellular melanin content

In order to evaluate the melanin inhibiting effect, B16F10 cells were treated with the test substance without stimulation with a-MSH. The chicken protein hydrolysate showed an inhibitory effect on the melanin synthesis and no significant cytotoxicity. The positive control was Arbutin and the melanin content was 80.1±1.5%. The melanin content of chicken protein hydrolysate was 90.7±1.4% at 4 mg/mL (Fig. 4a).

After 30 nM of α-MSH-induced melanogenesis in melanoma cells, chicken protein hydrolysate at concentrations between 1, 2, 4 mg/mL significantly inhibited melanin production, the melanin content was down to 84.7± 4.8, 81.6±1.7 and 70.3±3.4% (Fig 5a). The melanin content of arbutin was 66.8±3.3% with α-MSH stimulation. The color of B16F10 cell pellets treated with different concentrations and with or without α-MSH stimulation are shown in Fig 4b and 5b.

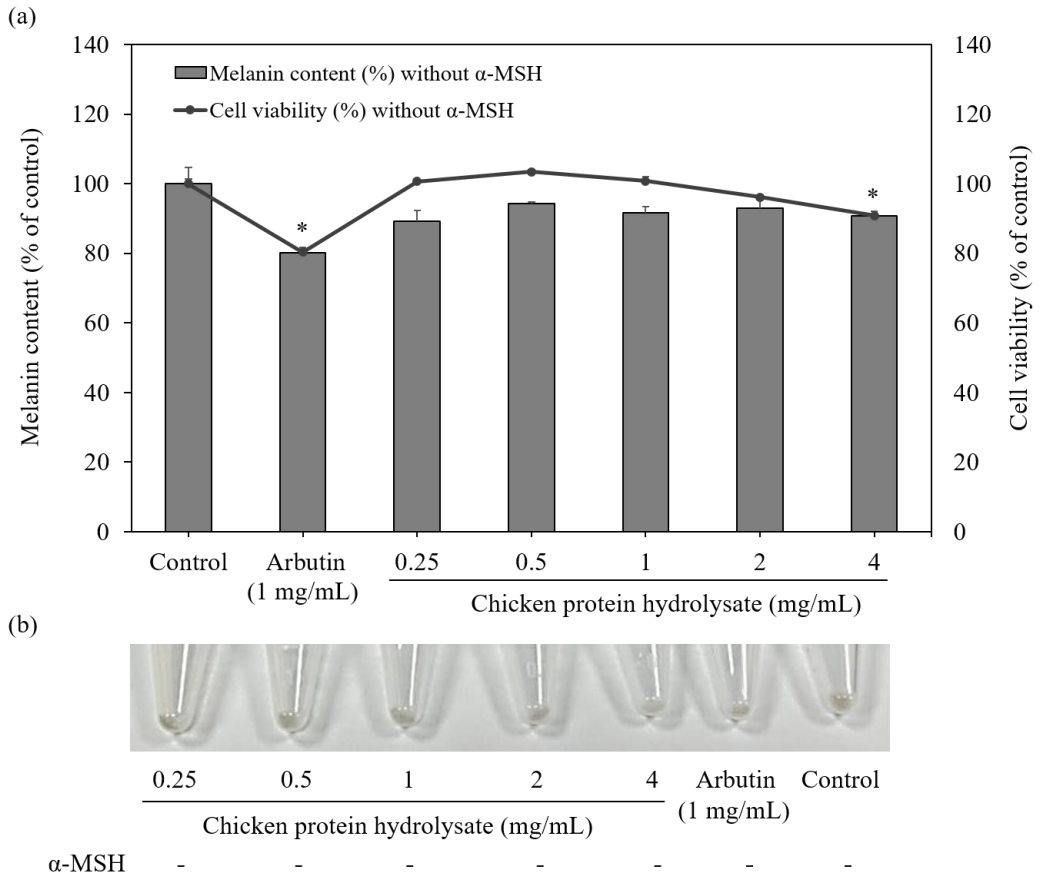

Fig. 4. *Melanin content of chicken protein hydrolysate on B16F10 without α-MSH-induced melanin. Negative control: DI water, positive control: arbutin (1 mg/mL), and test substance: chicken protein hydrolysate. *p<0.05 compared to control.*

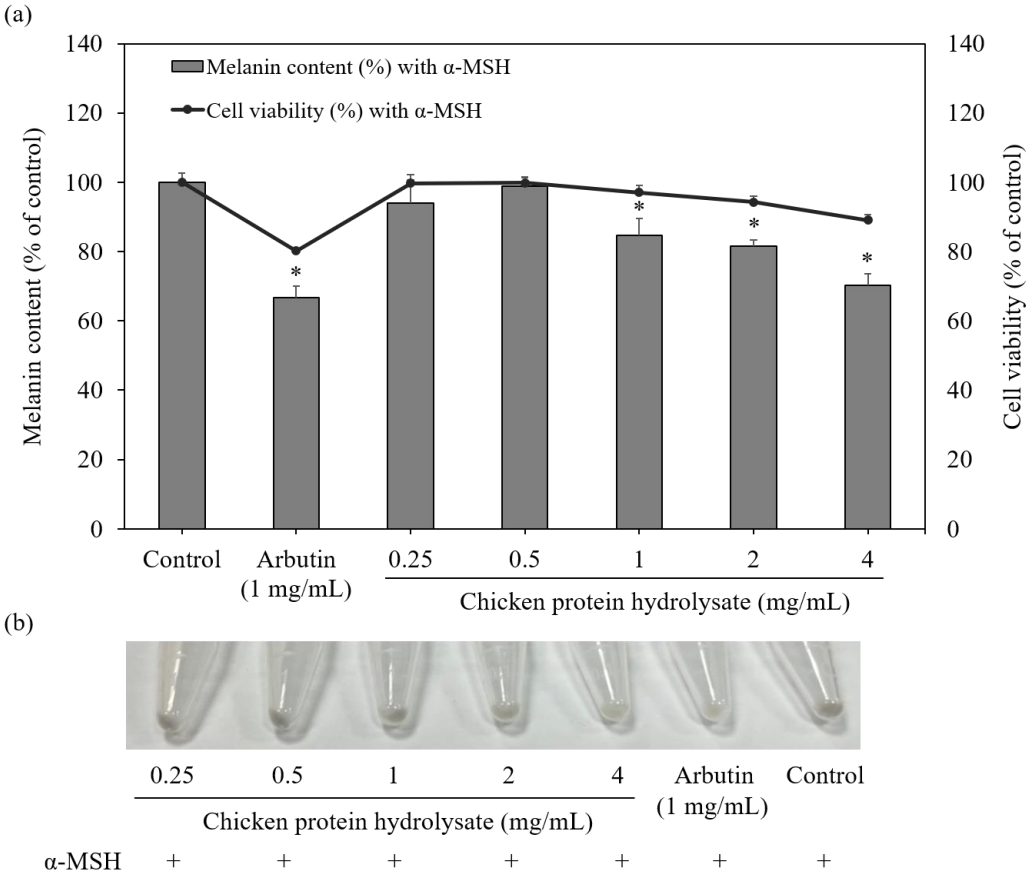

Fig. 5. *Melanin content of chicken protein hydrolysate on B16F10 with α-MSH-induced melanin. Negative control: DI water, positive control: arbutin (1 mg/mL), and test substance: chicken protein hydrolysate. *p<0.05 compared to control.*

CONCLUSION

In this study, we followed OECD TG 439 using a skin model as an alternative of animal testing. The results showed that chicken protein hydrolysate and collagen tripeptide are non-irritants.

In the cosmetics industry, collagen is valued for its availability, biocompatibility and biodegradability. The results showed that chicken protein hydrolysate and collagen tripeptide can enhance the synthesis of type I collagen to 99.3% and 129.4% and promote fibroblast proliferation to 62.2% and 22.1%.

Chicken protein hydrolysate and collagen tripeptide both have significant effects on collagen type I synthesis and promote the proliferation of fibroblasts. Chicken protein hydrolysate (4 mg/mL) has shown the ability to inhibit melanin production. Chicken protein hydrolysate can inhibit melanin production for 29.7% (with α-MSH stimulation).

According to these results, we can announce that chicken protein hydrolysate and collagen tripeptide both have high potential to use as raw material of cosmetics to anti-aging and whitening.

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Conflict of Interest. "The authors declared that there is no conflict of interest."

Authorship Contributions. Concept: H.Y.K., S.J., Y.K., Design: H.Y.K., S.J., Y.K., Data collection: H.Y.K., S.J., Y.K., Analysis or Interpretation: H.Y.K., S.J., Y.K., Literature Search: H.Y.K., S.J., Y.K., Writing: H.Y.K., S.J., Y.K.

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