



Research Article

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Preparation and toxicity of protein-calcium complex with phosphorylated human-like collagen

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ABSTRACT

In order to bind more amount of calcium, human-like collagen (HLC) was first phosphorylated by reacting with D-Glucose 6-phosphate disodium salt hydrate, and then the phosphorylated HLC (PHLC) was chelated with CaCl₂ to prepare the PHLC-Ca composite. The Optimum reaction condition for the PHLC-Ca was: pH 7.5, n_{CaCl₂} : n_{HLC} = 600, stirring for 1 h. The Cell Counting Kit-8 (CCK-8) assay indicates that HLC, PHLC and PHLC-Ca were safe to BHK21 and could stimulate the normal growth of BHK21.

Keywords: Optimum condition, toxicity of samples, human-like collagen, calcium

INTRODUCTION

Calcium plays a very important role in our daily life, which is one of the most abundant mineral in the human beings. 99% of the calcium is in bones and teeth, and the calcium in the blood and other body fluids, tissues accounted for only 1% of the total. Calcium is central to normal cell processes, because the tight regulation of intracellular calcium is crucial to homeostasis [1]. Calcium also regulates the activity of certain enzymes, such as adenosine triphosphatase, succinic acid dehydrogenase and lipase[2]. An adult daily reference intake is 800 mg calcium, while pregnant women intake is 1200 mg. Although the food contains abundant calcium, due to the unreasonable dietary structure, 90% of the people need to intake more calcium.

To enhance the bioavailability, numerous studies have been conducted. Many researches have been proved that reducing agents such as glucose and ligands such as amino acid, protein, will generally enhance the absorption of calcium[3]. Protein which is bioactive is likely to enhance calcium solubility and biocompatibility. The best protein for chelating substrate to bind calcium is collagen, because collagen is one composition of bone. In this research, HLC was severed as a new chelating substrate to bind calcium.

HLC is a giant molecule bio-protein which was produced by gene engineering. In the study, HLC was used as a good candidate, which is a recombinant protein produced by *E. coli*, containing human collagen's cDNA transcribed reversely from mRNA. The previous study demonstrated that it has several special characteristics which are significantly different from animal collagen, such as chemically defined structure, virus-free, biocompatibility, water solubility, and so on [4,5].

EXPERIMENTAL SECTION

Material

The human-like collagen (HLC, patent number: ZL01106757.8, Mr=97,000) was purchased from Xi'an Giant biogene technology Co.Ltd. 3-(N-morpholino) propanesulfonic acid (Mops) and D-Glucose 6-phosphate disodium salt hydrate were purchased from Sigma-Aldrich Co. (St. Louis, USA). Cell Counting Kit-8 (CCK-8) was purchased from Signalway Antibody Co. Ltd. Sephadex G-25 was purchased from GE Healthcare Bio-Science AB (Beijing, China). All other chemicals used were of analytical reagent grade and double distilled water was used throughout.

Preparation of PHLC

Lyophilised HLC were dissolved at a 50 μ M concentration in double distilled water, and then added D-Glucose 6-phosphate disodium salt hydrate which has equal weight amount with HLC at pH 8.0. The mixing solution was put in 65 °C water for 2 hours, then freeze-dried using a Freeze Dryer (SIM International Group, FD5-10, US). Lyophilised samples were incubated in saturated KI concentration at 55 °C for 10 hours. Dryheated samples were dissolved in double distilled water and through a gel chromatography equipped with a XK16/100 (mm/cm) Sephadex G-25 column (ATKA purifier, GE Healthcare, US) at a flow rate of 1.5 mL per min to remove the free reagents, and then lyophilised [5].

Effects of concentration of CaCl₂ on calcium binding

Lyophilised phosphorylated HLC was dissolved at a 20 μ M concentration in MOPS buffer solution (50 mM, pH 7.0). The HLC solution was mixed with a stirring bar to dissolve protein completely at room temperature (approximately 25 °C). 100 μ L, 300 μ L, 500 μ L, 600 μ L, 700 μ L, 800 μ L of CaCl₂ solution (0.5 M) ($n_{\text{CaCl}_2} : n_{\text{HLC}}$ were respectively 100, 300, 500, 600, 700, 800) were respectively dropped slowly to 25 mL HLC solution. The reaction mixture was stirred at room temperature for 2 hours, next the mixing solution was filtrated through 0.45 μ m filter, and then through a gel chromatography like before to remove the free ion. The purified chelate was lyophilised in the end. The calcium contents of the chelate was measured by atomic absorption spectrophotometry (AAS; Perkin-Elmer, model 2380) after the chelate was digested by aqua regia.

Effects of pH on calcium binding

Lyophilised phosphorylated HLC was dissolved at a 20 μ M concentration in MOPS buffer solution (50mM). Every 25mL HLC solution was respectively regulated pH at 6.0, 7.0, 7.5, 8.0, 8.5. The HLC solution was mixed with a stirring bar to dissolve protein completely at room temperature. 500 μ L of CaCl₂ solution (0.5M) were dropped slowly to HLC solution. The reaction mixture was stirred at room temperature for 2 hours, next the mixing solution was filtrated through 0.45 μ m filter, and then through a gel chromatography like before to remove the free ion. The purified chelate was lyophilised in the end. The calcium contents of the chelate was measured by atomic absorption spectrophotometry after the chelate was digested by aqua regia.

Effects of time of reaction on calcium binding

Lyophilised phosphorylated HLC was dissolved at a 20 μ M concentration in MOPS buffer solution (50 mM, pH 7.0). The HLC solution was mixed with a stirring bar to dissolve protein completely at room temperature. 500 μ L of CaCl₂ solution (0.5 M) were dropped slowly to 25 mL HLC solution. The reaction mixture was stirred at room temperature respectively for 0.25, 0.5, 1, 1.5, 2 hours, next the mixing solution was filtrated through 0.45 μ m filter, and then through a gel chromatography like before to remove the free ion. The purified chelate was lyophilised in the end. The calcium content of the chelate was measured by atomic absorption spectrophotometry after the chelate was digested by aqua regia.

CCK-8 reduction assay

The lyophilized samples of HLC, PHLC, PHLC-Ca were dissolved in RPMI-1640 medium to the concentration of 5 μ M, 15 μ M, 25 μ M, 35 μ M and then filtered through a 0.22 μ m filter under sterilized conditions. Baby hamster kidney cells (BHK21) were cultured at a density of 1.0×10^4 cells/mL on 96-well plates (100 μ L/well). Cells were cultured at 37°C in an incubator with a 5% CO₂ and 95% air atmosphere at constant humidity. After incubation for 24 h, the medium in wells was discarded, then 100 μ L samples were respectively added to 96-well plates (100 μ L/well). After incubation for 12 h and 24 h, 10 μ L of CCK-8 was added to each well, after which the cultures were incubated at 37 °C for an additional 3 h. Absorbency of the solution was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) Reader (MODEL550, Bio-Rad, USA).

The relative cell growth (%) was calculated as:

$$\text{Relative cell growth} = (A_s - A_b) / (A_c - A_b) \times 100\%$$

A_s: sample (the medium with cells and samples, CCK-8)

A_c: control (the medium with cells, CCK-8)

A_b: blank (the medium without cells and samples, CCK-8)

RESULTS AND DISCUSSION

Optimum condition

The effects of concentration of CaCl_2 on calcium binding were shown in Fig. 1. From Fig. 1, it could be found that the contents of binding had shingle peak in this spectra. So the peak ($n_{\text{CaCl}_2} : n_{\text{HLC}} = 600$) is the optimum condition for concentration of CaCl_2 .

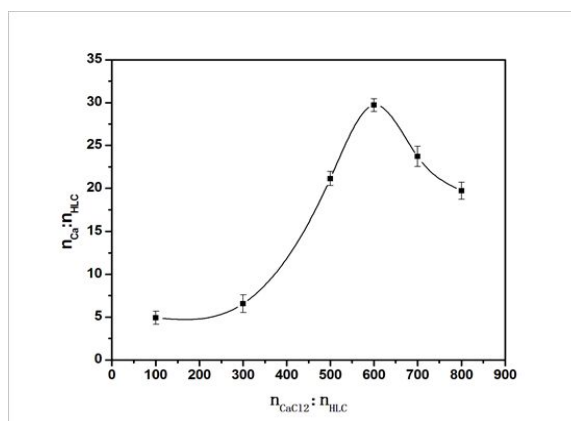


Fig.1 Effects of Concentration of CaCl_2 on Calcium Binding

The effects of pH on calcium binding were shown in Fig. 2. Obviously the curve of different pH with different amount of binding was a peak shape. It could also be seen from Fig. 2 that when pH was 7.5, the contents of binding was maximal. Thus pH 7.5 was optimum pH condition.

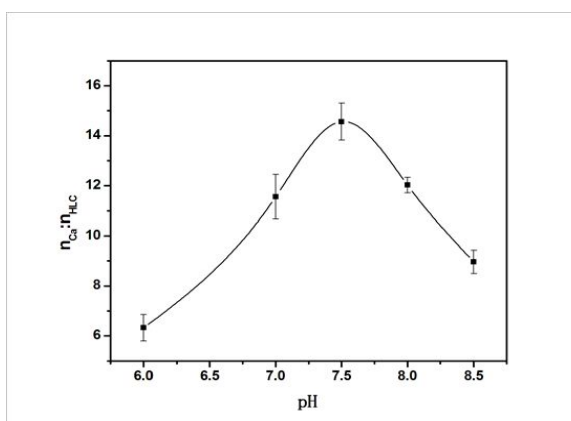


Fig. 2 Effects of pH on Calcium Binding

Fig. 3 showed the effects of time of reaction on calcium binding. In Fig. 3 when the time of reaction was 1 h, the increase of binding amount reached plateau. Thus the optimum time of reaction was 1 h.

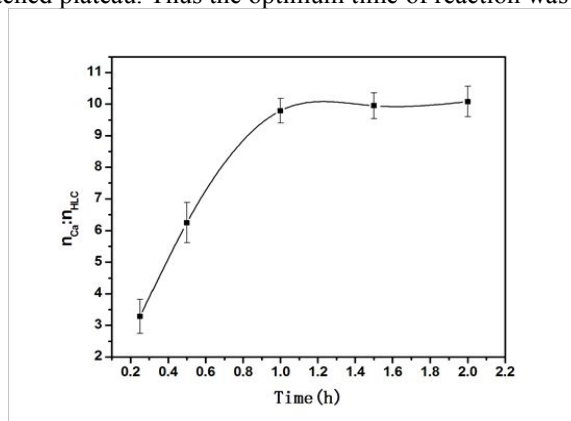


Fig. 3 Effects of Time of Reaction on Calcium Binding

The calcium contents of protein-calcium complex with native human-like collagen ($n_{Ca} : n_{HLC} \approx 7$) was lower than protein-calcium complex with phosphorylated human-like collagen ($n_{Ca} : n_{HLC} = 31.26$) at Optimum condition (pH 7.5, $n_{CaCl_2} : n_{HLC} = 600$, time of reaction 1 h) .

Cell Counting Kit-8 (CCK-8) reduction assay

The CCK-8 assay has been used to rapidly measure the toxicity of different substances in cell cultures. The effect of HLC, PHLC and PHLC-Ca on BHK21 viability was determined by CCK-8 assay for 12h and 24h (Fig. 4-5). Free HLC could promote BHK21 proliferation, and following the increase of concentration of HLC, the BHK21 proliferation promoted obviously. Compared with the control, P-HLC and PHLC-Ca triggered a significant increase in the number of BHK21 at 12 h and 24h. Therefore, HLC, PHLC and PHLC-Ca were safe to BHK21 and could stimulate the normal growth of BHK21, which can be a good calcium preparation to human beings for calcium supplement.

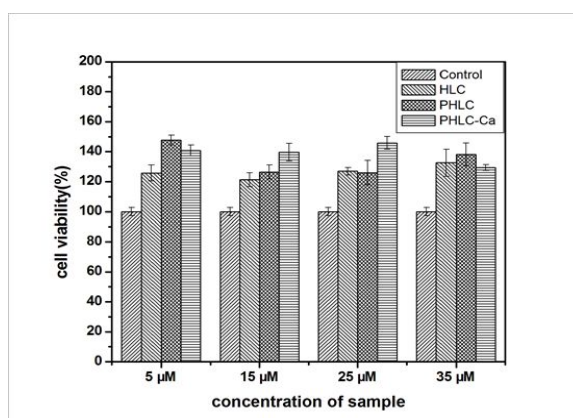


Fig. 4 Cell Viability in HLC, PHLC and PHLC-Ca After Culture for 12 h

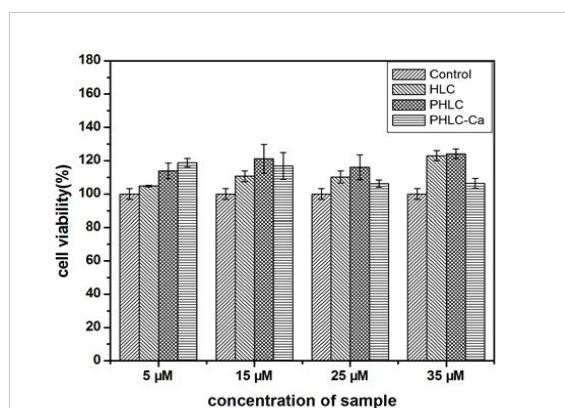


Fig. 5 Cell Viability in HLC, PHLC and PHLC-Ca After Culture for 24 h

CONCLUSION

D-Glucose 6-phosphate disodium salt hydrate provides a convenient method for the introduction of phosphate groups into human-like collagen under mild conditions. The phosphate group in human-like collagen makes the binding with calcium easily and quickly. Under the Optimum condition (pH 7.5, $n_{CaCl_2} : n_{HLC} = 600$, time of reaction 1 h), the amount of binding can reach to $n_{Ca} : n_{HLC} = 31.26$, which is more than the amount of binding native HLC with $CaCl_2$. The CCK-8 assay indicates that HLC, PHLC and PHLC-Ca were safe to BHK21 and could stimulate the normal growth of BHK21.

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