Components of chicken egg white extract smaller than 3 kDa in size promote 293T cell proliferation

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Received: 8 December 2014 / Accepted: 23 March 2015 / Published online: 5 November 2015
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Abstract We previously found that chicken egg white extract could promote cell survival and proliferation. In the present study, we further separated this extract into its components to identify those primarily responsible for promoting cell proliferation. Components of differing molecular weight were separated from chicken egg white extract by ultrafiltration and 293T cell cultures were supplemented with various concentrations. The effects on cell proliferation were subsequently determined by a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega). We demonstrate that components from chicken egg white smaller than 3 kDa in size are able to function as

active ingredients promoting cellular proliferation. This discovery may identify a new and convenient additive for cell culture media to promote cell growth and proliferation.

Keywords Chicken egg white extract · Active ingredients · Cell proliferation · Separation

Introduction

Eggs are one of the most economical sources of animal protein and possess the highest nutritional content. Egg white proteins contain many types of proteins, including ovalbumin, ovotransferrin, ovomucoid and lysozyme, which constitute 54, 13, 11 and 3.5 % of the egg white, respectively (Besler and Mine 1999), and have unique biological activities. A previous study (Mizunoya et al. 2015) reported significant proliferative activities of C2C12 cells in response to the addition of 10^{-5} – 10^{-4} mol/L ovalbumin or ovomucoid. In addition, ovotransferrin decreased C2C12 cell proliferation, whereas lysozyme had no significant effects on the proliferation of these cells. Lee et al. (2013) reported on the fractionation of egg white using tandem ion exchange chromatography (strong anion and strong cation exchange), and seven fractions were assessed for associated bioactivity, with four fractions at various protein concentrations possessing proliferative bioactivity that exceeded the FBS control.

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However, there is no report to date on <3 kDa components of chicken egg white that can promote cell proliferation. Some literature references have reported the sequence of chicken egg white mRNA, and the complete sequence of chicken ovalbumin mRNA is 1859 residues long. Chicken egg white contains an internal signal sequence of mature ovalbumin comprising residues 229–276 that can be recovered as a tryptic fragment (McReynolds et al. 1978; Lingappa et al. 1979). Our previous studies have found that chicken egg white can function in the maintenance of cell activity (Ruan et al. 2012), and our present study found that components in chicken egg white extract smaller than 3 kDa in size can promote cell proliferation and survival. This discovery may identify a new and convenient additive for cell culture media that promotes cell growth and proliferation.

We isolated chicken egg white, yolk and whole-egg extracts and found that both the egg white and whole-egg extracts could promote cell proliferation. Further analyses found that this function resides mainly in the egg white extract. Thus, chicken egg white extract is easily obtained and has the ability to promote cell proliferation, yet the specific components that mediate this function remain unknown. To address this, we conducted intensive studies on these extracts. Using ultrafiltration, chicken egg white extract was divided into the following fractions based on molecular weight: >50, 10–50, 3–10 and <3 kDa. The different fractions were supplemented at various concentrations to 293T cultures, and the effects on cellular proliferation were subsequently determined.

Materials and methods

Preparation of chicken egg extracts

The egg white and yolk of White Leghorn chicken eggs were isolated in a sterile manner. An equal volume of lysis buffer (50 mM NaCl, 5 mM MgCl₂, 100 mM HEPES pH 8.2, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail, purchased from Sigma, St. Louis, MO, USA) was added to the egg white; the lysis buffer was added to the egg yolk at a 1:3 yolk to buffer ratio. The solutions were fully mixed and stored at 4 °C. All reagents were of analytical purity and were manufactured in China. After 3 days, the solutions were

centrifuged, and the supernatants were stored at 4 °C to produce egg white and egg yolk extracts. To generate the whole-egg extract, a separate chicken egg was isolated in a sterile manner, lysed using a 1:2 egg to lysis buffer ratio, fully mixed and stored at 4 °C; after 3 days, the solution was centrifuged, and the supernatant was stored at 4 °C. The protein concentrations of the extracts were measured using the Bradford method (kits purchased from Shanghai Biyuntian Biological Co., Ltd., Shanghai, China). The extracts were then labeled (protein concentration, time of preparation), aliquoted and stored at –20 °C for later use. To generate a stable standard for this natural product, a minimum of six charges were used in our experiments.

Permeabilization of 293T cells

293T cells were purchased from the Kunming Cell Bank of Chinese Academy of Sciences (Yunnan Province, China). Because 293T cells are a standard cell line, a study using these cells is broadly representative. Permeabilization was performed as previously described (Taranger et al. 2005; Freberg et al. 2007), with some modifications. Briefly, 5×10^5 293T cells were washed with 500 µL HBSS and resuspended in 475 µL of ice-cold HBSS. The tubes were then incubated for 2 min at 37 °C, and 25 µL of streptolysin O (SLO) was added to a final concentration of 500 ng/mL. The samples were then incubated at 37 °C for 30 min, with the cells being mixed once at the midpoint of the incubation. Of note, the optimal concentration and incubation time for SLO was adjusted for each batch of SLO used. To each sample, 1 mL of ice-cold HBSS was added followed by centrifugation at $120 \times g$ for 5 min; 500 µL of HBSS was added as a negative control to one tube of permeabilized cells. In separate tubes, 500 µL chicken egg white extract, 500 µL egg yolk extract, 500 µL whole-egg extract, or 500 µL normal culture medium was added. 25 µL ATP regeneration system (ATP:GTP:creatine kinase:phosphocreatine in a 1:1:1:1 ratio from each separate stock, purchased from Sigma) and 20 µL of a 25 mmol/L nucleotide triphosphate (NTP) mixture had been previously added to the extracts. The samples were incubated at 37 °C for 30 min, with a single mixing step at the midpoint of the incubation. The contents of the five tubes were then transferred to 6-well culture plates, and the surviving cells were photographed on the following day.

SDS-PAGE electrophoresis

The chicken egg white extract and components greater than 3 kDa were prediluted; components smaller than 3 kDa were not prediluted. An aliquot of 50 μ L of each component was added to 50 μ L 2 $\times$ loading buffer, and the samples were boiled for 5 min at 100 °C. Ten microliters of each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V until the samples reached the bottom of gel. The concentration of the upper gel (stacking gel) was 5 %, and that of the lower gel (separating gel) was 12 %. The gel was stained with Coomassie Brilliant Blue for 3 h, destained overnight and photographed.

Separation of different chicken egg white extract components

Chicken egg white extract was separated using ultrafiltration tubes (purchased from Millipore) (molecular weight cut-offs of 50, 10 and 3 kDa) with a processing capacity of 15 mL. A total of 15 mL of chicken egg white extract was applied to the ultrafiltration tube, which was centrifuged at 4000 rpm for 30 min, separating components smaller than 3 kDa in size. The remaining extract was then diluted with lysis buffer to 15 mL, applied to a 10 kDa ultrafiltration tube and centrifuged at 4000 rpm for 30 min to obtain a 3–10 kDa fraction. Components greater than 10 kDa in size were diluted with lysis buffer to 15 mL and separated using a 50 kDa ultrafiltration tube to obtain 10–50 kDa and greater than 50 kDa fractions. At each step, we diluted the unfiltered extract with lysis buffer to the original volume of 15 mL.

Detection of cell proliferation

A suspension of 293T cells was adjusted to a concentration of 1×10^5 cells/mL, and 100 μ L PBS was added to the outer perimeter wells of a 96-well plate (36 wells in total) to prevent evaporation from the wells. Then, 50 μ L cells (5×10^3 cells per well) was added to the internal wells of the plate. Next, 50 μ L of extract was added to achieve a final concentration of 50 %. If the extract was prediluted with medium to create a 50 % final concentration, then 50 μ L of the extract was added to each well for a final concentration of 25 % extract. Each extract was added

to 10 wells, and the plate was incubated at 37 °C for 3–4 days. A CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) was used to detect cell viability. Briefly, 20 μ L of reagent was added to each well followed by incubation at 37 °C for 3 h and detection at 490 nm using a colorimeter. The OD value (of the y-axis) is correlated to active cell number.

Statistical analysis

The data are presented as the mean $\pm$ SD. Groups were compared using one-way ANOVA with SPSS 17.0 statistical software. Then, pairwise comparisons were carried out using LSD. $P < 0.05$ was considered statistically significant.

Results

Effects of three different chicken egg extracts on cell survival and growth

The chicken egg white extract most strongly promoted 293T cell survival and growth, followed by the whole-egg extract. The egg yolk extract had the smallest effect on the growth of cells, though this effect was stronger than that of the control (HBSS) (Fig. 1). We then extended the incubation time with SLO, which increased cellular perforation, and did not reseal the resulting holes in the cell membranes with calcium chloride. By the second day after treatment, all of the cells treated with the egg white extract survived and were adherent, whereas only a portion of the cells treated with the whole-egg extract survived. The cells treated with the egg yolk extract showed the smallest number of surviving cells. The control (HBSS) cells were all dead. The experiments were repeated more than three times, with similar results. The survival rates of the cells treated with egg white extract were the same as for the cells treated with medium. The finding that the egg white extract better promoted cell growth warranted further studies.

SDS-PAGE results of chicken egg extracts

Protein concentrations were measured using the Bradford method according to the manufacturer's instructions, and the results are provided in Table 1.

Fig. 1 Results of cell survival after permeabilization and exposure to different extracts. ($\times 100$); **a** HBSS, **b** egg white extract, **c** egg yolk extract, **d** whole-egg extract, **e** normal culture medium

Table 1 Protein concentration (mg/mL) of three extracts

Extract	Protein concentration (mg/mL)
Chicken egg white extract	24
Greater than 3 kDa components	31
Smaller than 3 kDa components	0.14

The results of electrophoresis showed that the chicken egg white extract contains three main bands: 90, 40 and 12 kDa (Fig. 2).

SDS-PAGE electrophoresis revealed no proteins among the components smaller than 3 kDa. The

components greater than 3 kDa showed the same protein bands as the chicken egg white extract.

Effects of components of chicken egg extracts at 50 % final concentration on 293T cell culture after 2 days of incubation (Fig. 3)

As the figure shows, the <3 kDa fraction of chicken egg white extract (50 % final concentration) promoted cell proliferation more strongly than the chicken egg white, egg yolk or whole-egg extract; the effects of the >3 kDa fraction were less strong than the medium in Fig. 3a. As the egg white extract promoted cell proliferation more strongly than either the egg yolk or whole-egg extract,

Fig. 2 Electrophoresis of the different components of chicken egg white extract; 1 protein molecular weight standards (from Thermo), 2 chicken egg white extract, 3 greater than 3 kDa components in chicken egg white extract, 4 smaller than 3 kDa components in chicken egg-white extract

we therefore focused our studies on the egg white extract. As shown in Fig. 3b, the <3 kDa fraction of chicken egg white extract (50 % final concentration) promoted cell proliferation more strongly than the lysis buffer. After ultrafiltration, the lysis buffer and other small molecules were separated from the egg white extract. Because the <3 kDa fraction showed important effects in this study, we demonstrated that the lysis buffer had no effect on cell proliferation. Through this experiment, we identified that the active components in the <3 kDa fraction do not include the lysis buffer.

Effects of components of chicken egg extracts at 50 % final concentration on 293T cell culture after 3 days of incubation (Fig. 4)

As the figure shows, the <3 kDa chicken egg white extract fraction (50 % final concentration) promoted cell proliferation to a lesser extent than the medium did but more strongly than any other fractions or the balanced salt PBS or HBSS controls.

Effects of components of chicken egg extracts at 50 % final concentration on 293T cell culture after 4 days of incubation (Fig. 5)

As the figure shows, the <3 kDa fraction of chicken egg white extract (50 % final concentration) promoted cell proliferation to a lesser extent than the medium

Fig. 3 Effects of various components of chicken egg extracts (50 % final concentration) on proliferation of 293T cells at an incubation time of two days ($n = 10$) (a $*P < 0.01$ when compared with other groups. b $*P < 0.01$ when compared with the other groups, except for the PBS group). The OD value correlated with the number of viable cells, with a high OD accompanying a high viable cell number. The figure shows relative quantification; for 5×10^3 cells per well cultured in medium for 2 days, the OD value is approximately 1. However, as each batch experiment presents an inherent error, the OD value was not necessarily 1 every time. As each figure has its own internal control, OD values should be compared within the same panel rather than among different panels. The use of the medium condition (DMEM-F12 medium supplemented with 10 % FBS) led to higher cell growth than all other conditions

did but more strongly than the other fractions or the balanced salt PBS or HBSS controls, with the exception of the 3–10 kDa fraction.

Effects of different components of chicken egg extracts at 25 % final concentration on 293T cell culture after 3 days of incubation (Fig. 6)

As shown in Fig. 6, the <3 kDa fraction from chicken egg white extract (final concentration of 25 %) promoted cell proliferation more strongly than did the commonly used medium ($P < 0.01$ in Fig. 6a,

Fig. 4 The effects of individual components of chicken egg white extract (50 % final concentration) on proliferation of 293T cells when incubated for 3 days ($n = 10$) ($*P < 0.01$ when compared with the other groups, except the medium group). The use of the medium condition (DMEM-F12 medium supplemented with 10 % FBS) led to higher cell growth than all other conditions

Fig. 5 The effects of different fractions of chicken egg white extract (50 % final concentration) on proliferation of 293T cells when incubated for 4 days ($n = 10$) ($*P = 0.738$ compared with the 3–10 kDa group, $P < 0.01$ compared with the other groups). The use of the medium condition (DMEM-F12 medium supplemented with 10 % FBS) led to higher cell growth than all other conditions

$P < 0.05$ in Fig. 6b). This result identifies the <3 kDa components in chicken egg white extract as a potential additive for cell culture media to better promote cell proliferation than the medium commonly used. Compared to the various components of chicken egg extracts, the effect of lysis buffer was poor.

Various components of chicken egg extracts (25 % final concentration) have different effects on proliferation of 293T cells when cultured for 3 days (Fig. 7)

As observed in Fig. 6b, the <3 kDa fraction of chicken egg white extract (25 % final concentration) promoted

Fig. 6 The effects of different fractions of chicken egg white extract (25 % final concentration) on proliferation of 293T cells when incubated for 3 days ($n = 10$) (a $*P = 0.01$ compared with the 3–10 kDa group, $P < 0.01$ compared with the other groups. b $*P < 0.05$ compared between any two groups). The OD value has a relationship with cell proliferation; a higher OD value indicates a greater number of the viable cells

cell proliferation more strongly than the commonly used medium ($P = 0.012$). This result identifies the <3 kDa components in chicken egg white extract as a potential additive for cell culture media to better promote cell proliferation than the medium commonly used.

Different concentrations of different components of chicken egg white extract have different effects on proliferation of 293T cells (Fig. 8)

As shown, the <3 kDa fraction from chicken egg white extract promoted cell proliferation more strongly than the commonly used medium ($P < 0.01$). Therefore, this result demonstrates our ability to isolate <3 kDa components from chicken egg white extract for use as an additive in cell culture media to promote cell proliferation.

Fig. 7 Different effects of various components of chicken egg extracts (25 % final concentration) on cell proliferation when incubated for 3 days ($n = 10$). (* $P = 0.012$ compared with the medium group, $P < 0.01$ compared to the other four groups)

Fig. 8 Different effects of various concentrations of each egg white extract components on proliferation of 293T cells when incubated for 3 days ($n = 10$) (* $P = 0.408$ compared to <3 kDa (50 %), $P < 0.01$ compared to the other five groups)

Discussion

Chicken egg yolk is the largest component of the egg, and the yolk membrane functions similarly to a cell membrane. The egg white and eggshell, which have roles in nutrition and protection, are formed from oviduct secretions. We prepared chicken egg white, egg yolk, and whole-egg extracts, used them to treat 293T cells, and observed the effects on cell survival and growth. The results showed that the chicken egg white extract contains substances that promoted 293T cell survival and proliferation. The effects were significantly different than those observed with the HBSS control. We assume that these substances are proteins, peptides or small molecules in the chicken egg. In our follow-up studies, we found that chicken egg white extract can promote cell survival.

A previous study (Azuma et al. 1989) reported that an acidic extract of egg white stimulated DNA synthesis and proliferation in BALB/c 3T3 cells, and the authors reported that the activity of the egg white extract could be divided into two fractions of approximately 7 and 2 kDa. The activity of both fractions was attributed to (poly)peptide compounds because the activity of each was lost by pronase digestion. However, there is no report in the literature of <3 kDa components of chicken egg white promoting cell proliferation. Although we do not know the active component or whether it is a known molecule, the <3 kDa components of chicken egg white are easily separated and can be obtained in a large volume. Thus, they constitute a potential additive for cell culture media to improve cell proliferation.

In this study, we found that chicken egg white extract could promote the growth and proliferation of 293T cells, though we did not identify the specific factor(s) mediating this effect. We separated the chicken egg white extract into different fractions of >50 , 10 – 50 , 3 – 10 and <3 kDa and cultured 293T cells with the different fractions at different final concentrations. The results showed that the <3 kDa fraction is able to promote cell proliferation. When the final concentration of the <3 kDa fraction was 25 %, a stronger effect on proliferation was observed compared to the medium alone. At a final <3 kDa component concentration of 50 %, the final medium concentration was 50 % with an FBS concentration 5 % lower than in the medium supplemented with 10 % of FBS; thus, cell proliferation activity was not as high as in the medium. However, when the final concentration of the <3 kDa components was 25 %, that of the medium was 75 % with a final FBS concentration of 7.5 %. The <3 kDa components promoted cell proliferation activity better than the medium at 10 % FBS. At the final concentration of 25 %, the <3 kDa components at 7.5 % of FBS promoted stronger cell proliferation role than the medium at 10 % of FBS. These results suggest that the <3 kDa components of chicken egg white extract are a potential additive for cell culture media, better promoting cell proliferation than media supplemented with 10 % FBS. We speculate that the components of chicken egg white extract smaller than 3 kDa contain some growth factors that can be used to promote 293T cell proliferation.

In our cell proliferation assays, we added the extracts at final concentrations of 50 and 25 %, and the

293T cells were subsequently cultured for 2, 3, or 4 days. Furthermore, due to the cumbersome steps associated with traditional MTT assays, we used an imported cell viability detection kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) that simply requires the addition of 20 μ L of reagent per well, incubation at 37 °C for 3 h, and detection at 490 nm using a colorimeter. This simplified the procedure, removing the need to aspirate the supernatant or to dissolve the precipitate. After culturing, a colorimeter was used for direct measurements, making the results more accurate and more reproducible.

Conclusions

Our results indicate that components smaller than 3 kDa in chicken egg white extract at a final concentration of 25 % more strongly promote 293T cell growth than culture medium. We can consider now isolating the components of chicken egg white extract smaller than 3 kDa for use as cell medium additives to promote cell growth and proliferation.

Acknowledgments This work was supported by National Support Program (2014BAI01B01) and Yunnan Provincial Science and Technology Project (2013CA005). We thank American Journal Experts for assisting in the preparation of this manuscript.

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