Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma

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Abstract

Sericin, a constituent of the silkworm cocoon, was added to the culture of four mammalian cell lines: murine hybridoma 2E3-O, human hepatoblastoma HepG2, human epithelial HeLa and human embryonal kidney 293 cells. The proliferation of all cell lines was accelerated in the presence of sericin. The hybridoma cell line was further studied. The 2E3-O cell line was so well adapted to serum-free medium that both the proliferation rate and maximum cell density in serum-free ASF103 medium were higher than in RPMI medium supplemented with all lots of FBS tested, and this proliferation was stimulated by the addition of sericin in a dose-dependent manner. Stimulation was observed at sericin concentrations from 0.01 to 0.1%, although 1% sericin was severely harmful to the culture. In comparison with bovine serum albumin (BSA), a widely used supplement in serum-free medium, sericin had an equivalent effect on the proliferation of the hybridomas and sericin additively stimulated the proliferation with BSA. Although heat easily denatures and inactivates most proteins, the activity of sericin was not affected by autoclaving. In a similar manner to the silkworm-derived sericin, recombinant sericin synthesized in *E. coli* also stimulated the hybridoma proliferation, irrespective of whether it was autoclaved or filtered. Since BSA is obtained from bovine serum and the risk of infections such as bovine spongiform encephalopathy cannot be eradicated, sericin derived from insects could be a preferable culture medium supplement for stimulating the proliferation of mammalian cells.

Abbreviations: BSA – bovine serum albumin; FBS – fetal bovine serum; MoAb – monoclonal antibody.

Introduction

Most mammalian cells require serum or its replacement in the culture medium during culture. The sera used most frequently are fetal bovine serum (FBS) and calf serum and these are frequently contaminated with viruses, and even the risk of prions, or bovine spongiform encephalopathy (BSE), cannot be erased. Serum also contains unknown factors outside the operator's control in addition to a variety of identified factors, including proteins such as albumin etc. Therefore, for the recovery of cell products the presence of serum is the principal obstacle to purification and limits pharmaceutical acceptance of the product. Sera

vary from batch to batch and each cell type requires a different batch of sera, so several batches must be held on reserve simultaneously even in a single laboratory.

Serum-free medium has been developed in order to overcome these disadvantages of serum (Murakami, 1984). Albumin, the major component of serum along with globulin, is thought to be important as a carrier of lipids or minerals and is used at 1–10 mg ml⁻¹ in serum-free medium.

Chang et al. (1980) successfully developed serum free-medium in which several hybridoma cell lines proliferated and secreted their antibody at a comparable rate to those in the presence of serum. This serum-free medium was supplemented with insulin,

Figure 1. HPLC analysis of sericin. Sericin (a) or marker proteins (b) were dissolved in 0.2 M NaCl and 0.05 M Tris HCl buffer (pH 7.0) and then were loaded to Superdex 75HR column (Pharmacia, U.S.A.). Absorbance at 275 nm was measured. 1, BSA (67 kD); 2, Ovalbumin (43 kD), 3, Chymotrypsinogen A (25 kD), 4, Ribonuclease A (13.7 kD); 5, Aprotinin (0.65 kD).

which promotes the uptake of glucose and amino acids (Kelly et al., 1978), and transferrin, which binds iron to make it less toxic and more bio-available (Guilbert and Iscov, 1976).

Murakami et al. (1982) reported that serum-free medium containing insulin, transferrin, selenite and ethanolamine successfully stimulated the growth of various types of hybridoma and this supplement combination was named ITES.

Although these remarkable accomplishments have been achieved, none of the serum-free media can satisfy the needs of all types of cells. Each cell type requires a different recipe and the cultures vary in their requirements from origin to origin, and even within one class. Due to this multiplicity, novel factors inducing proliferation are desirable. The present study reports that the sericin protein could be useful as a culture medium supplement for various mammalian cell lines.

Figure 2. Effect of sericin on proliferation of several mammalian cell lines (a) hybridoma and (b) HeLa. (a) 20 000 hybridoma cells were cultured in ASF103 medium with or without sericin for 50 h and the cell densities were determined by counting in a hemocytometer under a phase contrast microscope using trypan blue exclusion. (b) 25 000 HeLa cells were seeded in 1 ml of DMEM medium supplemented with 10% FBS and cultured for five days.

Sericin and fibroin are the two major components of raw silk. Sericin constitutes about 20–30%, while

fibroin is the predominant component and minor components are 1% salt and 2% wax that presumably acts

Figure 2. (continued). Effect of sericin on proliferation of several mammalian cell lines (c) HepG2 and (d) 293 cells. (c) 50 000 HepG2 cells were seeded in 1 ml of SF-O2 medium and cultured for five days. (d) 77 500 293 cells were cultured for two days in DME-medium supplemented with 0.1% sericin (solid bar), with 10% FBS as a positive control (striped bar) or without either of them as a negative control (open bar).

as a water repellent for the cocoon (Fourier, 1979; Voegeli et al., 1993). Sericin, an albuminoid protein consisting of serine (30%) and other amino acids, is a gummy coating on raw-silk filaments and, in order to make the silk lustrous and semitransparent, it is removed by a treatment called degumming. The degumming treatment is essentially an alkaline scouring operation and is carried out at a temperature of

about 95 °C. In this process, sericin is degraded and solubilized in water. Although the boil-off solution which contains a high amount of sericin is used as a buffer agent for dye solutions, most sericin is disposed of.

Recently, several functions of sericin have been revealed and novel applications have been proposed. Minoura et al. reported that murine fibroblast L-929

Figure 3. Proliferation of hybridomas in the presence of different concentrations of sericin. (a) 20 000 hybridoma cells were cultured in ASF104 medium (BSA-free) with sericin for 110 h and the cell densities were determined by counting in a hemocytometer. (b) Growth curve of cultures in the presence (closed circles) or absence (open squares) of 0.1% sericin.

cells attached to films made of sericin and proliferated (Minoura et al., 1995). Sericin acts as antioxidant to inhibit tyrosinase and lipid peroxidation (Kato et al., 1998) and, since oxidative stress is postulated to induce carcinogenesis, dietary sericin could be a potent

inhibitor of carcinogenesis. Indeed, feeding diets containing sericin to ICR mice suppressed colon carcinogenesis induced by 1,2-dimethylhydrazine (Sasaki et al., 2000a). The consumption of sericin has also been suggested to enhance the bioavailability of several

Figure 4. Comparison of the sericin and BSA effects on hybridoma proliferation 15 000 hybridoma cells were cultured in ASF104 medium (BSA-free) for 72 h with sericin (shaded bars), with BSA (hatched bars), with sericin and BSA (closed bars) or without either of them (open bar). 1 indicates the negative-control culture; 2, 3 and 4 indicate cultures with 0.025, 0.5 and 1% sericin, respectively; 5 and 6 indicate cultures with 0.5 and 1% BSA, respectively; 7 and 8 indicate cultures with 0.5% sericin and 0.5% BSA, and with 1% sericin and 1% BSA, respectively.

metal ions in rats (Sasaki et al., 2000b). A recombinant sericin peptide had protective effects against freezing stress in *E. coli* (Tsujimoto et al., 2001). These results were obtained using sericin heated at 95 °C for 2 h during the preparation, suggesting that purified sericin could display equivalent activity after additional applications of heat such as autoclaving.

In the present study, it is reported that sericin induces the proliferation of several cell lines, including hybridoma cells, and is a candidate for a supplement for serum-free medium.

Materials and methods

Preparation of sericin

Sericin from the cocoons of *Bombyx mori* were prepared by the following procedures (Kato et al., 1998). Five liters of 0.2% sodium carbonate solution (pH 11–12) containing 100 g of the cocoon was heated at 95 °C for 2 h and then filtered through a glass microfiber filter (GF/A Whatman International Ltd., U.K.) in order to remove the fibroin. The filtrate was dialyzed against deionized water and dried by spray drying. The amino acid composition of the extracted sericin was analyzed using an amino acid analyzer (LC-10A, Shimazu, Japan). HPLC analysis was also performed in order to

reveal the condition of sericin. Sericin in 0.2 M NaCl and 0.05 M Tris HCl (pH 7.0) buffer was loaded into Superdex 75HR column (Pharmacia, U.S.A.) and absorbance at 275 nm was measured. The same lot of sericin was used for all experiments in this article.

The recombinant 152 amino acid sericin peptide was synthesized in *E. coli* transfected with pQEserT and prepared by the method of Tsujimoto (Tsujimoto et al., 2001). The peptide had a high proportion of hydrophilic amino acid residues (Ser, 45%; Gly, 16%; Thr, 11%; Asn, 11%).

Unless otherwise noted, sericin was sterilized by filtration.

Cell lines and culture conditions

The cell line 2E3-O, employed throughout this study, was a mouse hybridoma derived from a mouse myeloma, P3X63 AG8U.1, by electric fusion with mouse spleen cells (Makishima et al., 1992). The hybridoma was a producer of an IgG1 specific for a trinitrophenyl-hapten. The cells were cultured in serum-free ASF103, serum- and BSA-free ASF104 (Ajinomoto, Japan) or RPMI 1640 medium (Nissui, Japan) supplemented with 10% FBS (vol/vol), 20 mM HEPES, 0.2% NaHCO₃, 2 mM glutamine and 0.06 mg ml⁻¹ kanamycin.

Table 1. Amino acid composition of sericin

Amino	Molar percentage
Asp	18.0
Thr	8.4
Ser	32.2
Glu	4.6
Pro	0.6
Gly	15.7
Ala	5.3
Cys	<0.05
Val	3.6
Met	<0.05
Ile	0.7
Leu	1.1
Tyr	3.7
Phe	0.4
His	1.3
Lys	2.5
Arg	1.8

The HeLa and 293 cell lines were cultured in DMEM medium supplemented with 10% FBS (vol/vol). HepG2 was cultured in SF-O2 serum- and BSA-free media (Sanko Junyaku, Japan).

The cells were grown in 24 well plates (Asahi Techno Glass Co., Japan) at 37 °C in humidified air containing 5% CO₂. The numbers of viable and dead cells were determined by counting in a hemocytometer under a phase contrast microscope using trypan blue exclusion.

Results and discussion

Sericin accelerated the proliferation of four distinct mammalian cell lines

Mammalian cells are industrially cultured in order to produce bio-active proteins or vectors for gene therapy or to be used for cell therapy etc. Novel supplements for these mammalian cell lines are still required. Due to the variety of the demand, the effect of sericin was tested on several cell lines. The amino acid composition of the sericin used in this study was analyzed and is shown in Table I. HPLC analysis revealed that the hydrolyzed sericin ranged in the molecular weight from 6 kD to more than 67 kD (Figure 1).

The cell lines tested were HeLa human epithelial cells derived from a cervical carcinoma, the 293 cell line derived from human embryonal kidney transformed by sheared human adenovirus type 5 DNA, human hepatoblastoma HepG2 cells and the murine hybridoma 2E3-O cell line. Hybridomas are cultured for producing MoAbs which have been widely used as medicines and as reagents for clinical diagnosis.

In order to investigate the effect on proliferation, sericin was supplemented to the culture of the hybridomas for two days and then the cell density was determined. As shown in Figure 2a, culture in the presence of 0.1% sericin significantly increased the cell density, but culture in 0.03 and 0.01% did not.

As shown in Figure 2b, 0.03% sericin increased the population of the HeLa culture, but 0.3% sericin did not. Both 0.03% and 0.1% sericin significantly increased the population of HepG2, while 0.3% sericin had no influence (Figure 2c). In the absence of FBS, 293 cells did not proliferate, but the cells partly recovered their proliferation in the presence of 0.1% sericin (Figure 2d).

Addition of sericin to the medium therefore enhanced the proliferation in all the cultures tested, indicating that sericin could be a potent and widely effective supplement for mammalian cell culture.

It has been reported that the proliferation of fibroblasts on silk protein matrices containing sericin was stimulated (Minoura et al., 1995), and this also supports the potential of sericin for mammalian cell culture applications.

Effect of sericin on hybridoma proliferation

The hybridoma cell line 2E3-O was adapted to serum-free medium ASF103 (Ajinomoto, Japan) supplemented with 0.1% BSA. The cells proliferated more rapidly in the ASF103 medium than those in RPMI 1640 medium supplemented with any lot of FBS tested. The culture in ASF103 proliferated to 1 090 000 cells ml⁻¹, while the culture in RPMI 1640 with FBS only proliferated to 516 000 cells ml⁻¹. These results indicated that this cell line was well adapted to serum-free media, and therefore, further acceleration of the proliferation of the hybridomas in ASF103 medium in the presence of sericin seemed unlikely. This finding encouraged us to study the effect of sericin on the proliferation of the hybridoma cells.

Since sericin is accepted as an albuminoid protein, it might act on the same targets in cells as BSA, suggesting that the effect of sericin might be masked in the

Figure 5. Influence of the sterilization method on sericin activity. Sericin was sterilized by filtration (hatched bars) or autoclaving (closed bars) and added to the culture. 20 000 hybridoma cells were cultured for 72 h and the cell densities were determined.

presence of BSA. Therefore, to remove BSA activity, the culture was performed in ASF104 medium where BSA was absent.

Hybridoma cells were inoculated into ASF104, a BSA-free medium, supplemented with decimally diluted sericin and cultured for 110 h. The viable cell densities of the cultures in the presence of sericin at various concentration are shown in Figure 3a. The cell density in the negative control culture was $191\,000 \pm 10\,000$ cells ml^{-1} and in the presence of less than 0.001% sericin, there were approximately 200 000 cells ml^{-1} . In the presence of 0.01% sericin, the cell density was increased to $258\,000 \pm 26\,000$ cells ml^{-1} , while in the presence of 0.1% sericin the number was markedly increased to $324\,000 \pm 15\,000$.

However, viable cells were never detected in the culture supplemented with 1% sericin, indicating that the presence of 1% sericin was very harmful to the culture.

In order to analyze the growth profile in detail, the growth curves of the culture with or without 0.1% sericin were determined (Figure 3b). During the early stage of the culture (before day four), the proliferation rate of the culture in the presence of sericin was higher than that in the absence of sericin, while during the later stage (after day four), the proliferation rates of both cultures were similar and sericin did not affect

the maximum cell density in the culture. These results indicate that sericin shortens the induction period (lag phase) and the culture enters the logarithmic phase earlier than in the absence of sericin, i.e., the hybridoma cells in the culture with sericin begin to divide earlier than those without. In fact, a significant increase in the cell population for the one day culture with sericin was observed (data not shown).

Comparison between the sericin and BSA effect on proliferation

The increases in the populations supplemented with sericin were observed whether BSA was present or absent, but the increases in the BSA-free culture were more marked. This might suggest that sericin displayed effects on proliferation that overlapped with BSA and could serve as a substitute for BSA. However, as shown in Figure 2a, an additive effect of sericin with BSA should exist during culture in ASF103 containing BSA. In order to study this further, they were simultaneously supplemented to the culture. The viable cell densities after a three day culture period were measured (Figure 4).

Figure 4 demonstrates that both sericin and BSA increased the population of hybridomas in a dose-dependent manner and that the combination of sericin

Figure 6. Recombinant sericin peptide effect on hybridoma proliferation. 16 000 hybridoma cells were cultured in ASF104 medium (BSA-free) for 72 h with filtered sericin (shaded bar), with autoclaved sericin (hatched bar), with filtered BSA as a positive control (closed bar), or without supplementation as a negative control (open bar). The concentration added was 0.01%.

and BSA caused the largest increase in the population during the culture. These results indicate that sericin would be a suitable medium supplement for use instead of BSA, and that a combination of sericin and BSA would realize more successful cultures.

Activity of sericin after autoclaving

Although most proteins are easily denatured by heating, our sericin preparation was purified by a procedure which included treatment with boiling water (95 °C) as described in the Materials and Methods, suggesting that additional application of heat would not inactivate sericin. Due to this thermostability, sericin could be exposed to the temperature required for autoclaving (121 °C), which is much less labor intensive and less expensive, and has a much lower failure rate, than membrane filtration.

Sericin, sterilized by either autoclaving or membrane filtration, was added to the hybridoma cultures for three days. The viable cell densities are shown in Figure 5. In the presence of 0.1% sericin, the hybridoma population increased equally, irrespective of the sterilization method. This result indicates that autoclaving did not affect the sericin activity.

However, in the presence of 0.01% sericin, the population of the culture with autoclaved sericin was slightly less than with the filtered one. This result suggests that autoclaving may slightly degrade sericin, although the autoclaved sericin did increase the population significantly.

Recombinant sericin peptide increased the population

A serine-rich peptide consisting of 38 amino acids was identified as an internally repeated motif in sericin,

and comparison of the sequence in different positions established a consensus 38-mer repetitive sequence (Tsujimoto et al., 2001). A recombinant sericin gene consisting of four repeats of the consensus sequence was designed and the expression plasmid pQEserT was transfected into *E. coli* for synthesis of the sericin peptide (Tsujimoto et al., 2001). This peptide was extremely hydrophilic because it had a high proportion of hydrophilic amino acid residues (Ser, 45%; Gly, 16%; Thr, 11%; Asn, 11%). The purified recombinant sericin peptide was added to the hybridoma cultures and its effect was investigated (Figure 6). In the presence of 0.01% recombinant sericin, the hybridoma population increased equally, irrespective of the sterilization method, although less so than with BSA. This result indicates that the recombinant sericin peptide has the same effect on proliferation and could become an unlimited supply of the sericin supplement.

Both the recombinant sericin and the preparation from the silkworm increased the cell populations. These results confirm that the growth inducing effect was not due to any contaminants contained in the sericin preparation, but to the sericin itself.

Conclusion

Addition of sericin to the culture medium successfully accelerated proliferation in all four mammalian cell lines tested. The mitogenic activity of autoclaved sericin was comparable to that of filtered sericin, and the recombinant sericin peptide also increased the population of hybridoma cells. These results indicate that sericin could be a potent and widely effective supplement for mammalian cell culture.

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