Plant Protein Hydrolysates as a Supplement for Medium of Human Skin Fibroblast 1184 Cell Culture

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Specially dedicated to

My love, *Mehrnaz Alishiri*,

My beloved father, *Ali Kazemzadeh*, My beloved mother, *Farahnaz Nikpour*,

My dear sister, *Golnaz Kazemzadeh*, her husband, *Ramin Andalib*, her daughter, *Pargol Andalib*

My dear brother, *Amir Hossein Kazemzadeh*

and those who have guided and give moral support to me

throughout my journey of education

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ABSTRACT

Medium is the most important item in cell culture. Each medium consists of two main parts which are a basal medium and serum. The main source of the serum is come from an animal blood. The serum from animal blood has many disadvantages such as viral contaminations. Moreover, serum is very expensive. Serum-free media is the best alternative in solving this problem. Unfortunately, serum-free medium is not sufficient for the cell growth. Thus, some supplements must be added to serum-free medium. One of the most important sources of supplements is from plants which could reduce the cost. In this study different plant protein hydrolysates from soy, sesame, *Aloe vera*, rice and wheat have been tested against Human Skin Fibroblast (HSF) 1184. All protein hydrolysates were prepared through enzymatic hydrolysis using commercial enzymes of non-animal origin. These hydrolysates were characterized according to their solubility and peptide size. Different growth behaviours of HSF 1184 cells were observed when these hydrolysates were added in DMEM with and without Fetal Bovine Serum (FBS). Hydrolysates from exopeptidase enzymes such as Flavourzyme gave negative effect on HSF 1184 cell culture, while hydrolysates from endopeptidase enzymes were supplementary for HSF 1184 cell culture. Since plant proteins do not have all the necessary amino acids for HSF 1184 cell culture growth, they cannot be solely substituted with FBS. Depending on the enzyme used, the supplementation with hydrolysates corresponding to a high degree of hydrolysis and composition of peptides with small molecular size, led to different maximal cell density. This indicates the importance of enzyme specificity and consequently the nature of the released peptides. In conclusion, the best plant protein hydrolysates for supplementation into the complete medium for HSF 1184 growth were soy and *Aloe vera* which were hydrolysed by Alcalase.

ABSTRAK

Medium adalah bahan yang paling penting dalam pengkulturan sel. Setiap medium terdiri daripada dua bahagian yang utama iaitu medium asas dan serum. Sumber utama serum ialah daripada darah haiwan. Serum daripada sumber haiwan mempunyai banyak kekerangan termasuklah dicemari virus. Medium tanpa serum merupakan alternatif terbaik untuk menyelesaikan masalah ini. Namun medium tanpa serum tidak mencukupi untuk pertumbuhan sel. Oleh sebab itu, beberapa nutrien tambahan perlu dimasukkan di dalam medium tanpa serum. Salah satu sumber nutrien tambahan adalah daripada tumbuh-tumbuhan yang mungkin dapat mengurangkan kos. Dalan kajian ini protein hidrolisat dari beberapa tumbuhan berlainan seperti soya, bijan, lidah buaya, beras dan gandum telah dikaji terhadap sel fibroblast manusia (HSF 1184). Kesemua protein hidrolisat dihasilkan dengan kaedah hidrolisis protein menggunakan pelbagai enzim komersil dari sumber bukan haiwan. Protein hidrolisat dikategorikan mengikut ciri-ciri keterlarutan dan saiz peptida. Pertumbuhan HSF 1184 adalah berbeza dan bergantung kepada jenis hidrolisat yang dicampurkan ke dalam medium DMEM mengandungi FBS dan tanpa FBS. Hidrolisat daripada enzim *exopeptidase* seperti Flavourzyme memberikan kesan negatif terhadap pertumbuhan sel HSF 1184. Manakala hidrolisat dari enzim *endopeptidase* diperlukan untuk pertumbuhan HSF 1184. Oleh kerana protein tumbuhan tidak mempunyai semua asid amino yang diperlukan untuk perkembangan HSF 1184, maka ianya tidak boleh menggantikan FBS sepenuhnya. Bergantung kepada enzim yang digunakan, penambahan hidrolisat berkait dengan ketinggian darjah hidrolisis dan molekul peptida bersaiz kecil akan membezakan nilai ketumpatan maksima sel. Ini menunjukkan betapa pentingnya pemilihan jenis enzim dan kesannya terhadap pembebasan peptida. Kesimpulannya, tumbuhan terbaik dalam kajian ini untuk membekalkan protein hidrolisat ke dalam medium lengkap bagi pertumbuhan HSF 1184 ialah soya dan lidah buaya yang dihidrolisis menggunakan Alcalase.

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LIST OF ABBREVIATIONS

Ala	-	Alanine
Arg	-	Arginine
Asn	-	Asparagine
Asp	-	Aspartic acid
A-tocopherol	-	Vitamin E
B-ME	-	B-Mercaptoethanol
BSA	-	Bovine Serum Albumin
BSE	-	Bovine Spongiform Encephalopathy
BVDV	-	Bovine Viral Diarrhea Virus
cAMP	-	cyclic Adenosine Monophosphate
CHO	-	Chinese Hamster Ovary
CTX	-	Cholera Toxin
Cys	-	Cysteine
DMEM	-	Dulbecco's Modified Eagle's Medium
Eagle's MEM	-	Eagle's Minimal Essential Medium
ECVAM	-	European Centre for the Validation of Alternative Methods
EGF	-	Epidermal Growth Factor
ELISA	-	Enzyme-linked Immunosorbent Assay
ESAC	-	ECVAM Scientific Advisory Committee
ESC	-	Embryonic Stem Cells
FBS	-	Fetal Bovine Serum
GCCP	-	Good Cell Culture Practice
Gln	-	Glutamine
GLP	-	Good Laboratory Practices
Glu	-	Glutamic acid
Gly	-	Glycine
GMP	-	Good Manufacture Practices
His	-	Histidine
Ile	-	Isoleucine
ITS supplements	-	Insulin, Transferrin and Selenium
Leu	-	Leucine
Lys	-	Lysine
Met	-	Methionine

NGF	-	Nerve Growth Factor
PDGF		Platelet-derived growth factor
Phe	-	Phenylalanine
PIs	-	Protease Inhibitors
PL	-	Platelet Lysates
Pro	-	Proline
Ser	-	Serine
T3	-	Triiodothyronine
Thr	-	Threonine
TGF-β		Transforming growth factor beta
Trp	-	Tryptophan
Tyr	-	Tyrosine
Val	-	Valine
Vitamin A	-	Retinoic acid

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CHAPTER 1

1. MAMMALIAN CELL CULTURE

1.1. Introduction

Mammalian cell culture has a bright yet changing future. Many challenges remain and new areas will open. The cell culture technologist will continue to work with cell biologists, biomaterials scientists, clinicians and regulatory authorities to produce effective and safe products to patients. In all areas, the development and application of protein-free media remains a priority to assist purification and ensure the use of biologically safe raw materials. Process intensification, particularly in generating gene therapy viral vectors and in recombinant protein and antibody production remains an important issue to generate sufficient material and lower production costs. In the field of recombinant protein production cell culture-based systems face competition from developments in emerging technologies such as production in transgenic animals. Virus production, whether for vaccines or for gene therapy, will however, inevitably be produced in some type of mammalian cell.

1.2. History

The period from 1880 to the early 1900s saw the first development techniques to study the behaviour of cells *in vitro* (An artificial environment outside the living organism). Although Harrison is normally accredited with the development of cell culture as a scientific tool, he described his own work as an extension of Wilhelm Roux (Keshishian, 2004). Both these scientists were interested in studying specific forms of cellular differentiation during embryo development. Roux however was not concerned with the multiplication of cells in culture. Harrison's "hanging drop" experiment enabled observation of the growth of nerve cells from the original explant and was able, with care, to maintain sterile growth for up to four weeks, which stimulated an expansion of interest in the science of *in vitro* cell growth and development.

Burrows established mammalian cell culture using chick embryos as the source of cells grown in the presence of plasma clots using Harrison's method (Bonassar, 1998). A significant development made by his group was the demonstration of the principle of media exchange and sub-culture. Burrows and other workers demonstrated growth of epithelial cells, connective tissue and a variety of tumour cells. Continuous passage of cells demonstrated by Ebeling and others led to the conclusion that somatic cells could survive indefinitely *in vitro* if media was replaced and conditions were appropriate (Bonassar, 1998).

The first permanent cell line was developed by Earle in 1943 from subcutaneous mouse tissue. Cell cultures were propagated continuously (designated strain L) and were shown to be morphologically (the study of the form or shape of an organism or part thereof) quite different from the original tissue. Thus it was shown that "transformed" cell lines could be developed. The first human "transformed" cell line was the HeLa cell (cell type in an immortal cell line used in scientific research), derived from a cervical carcinoma (Bonassar, 1998).Other techniques that came to be important for both small-scale and large-scale cell culture were developed during the 1950s and 1960s. These included the use of trypsin (digestive enzyme which breaks down proteins in the small

intestine) to permit sub-culture of attached cells from one flask to another, developments in cell culture vessels and bioreactors, methods of cell cryopreservation and developments in cell culture media formulations.

1.3. Mammalian Cell Culture Application

In the twentieth century, mammalian cell culture developed from its infancy, to providing a vehicle for viral vaccine production and most recently to produce monoclonal antibodies and other recombinant proteins. As we entered the new century, many more biopharmaceuticals, produced by mammalian cell culture will become available. Cell culture technology will play an important role in the emerging fields of gene therapy and tissue engineering.

The animal cell cultures are used for a diverse range of research and development. These areas are:

(a) Production of antiviral vaccines, which requires the standardization of cell lines for the multiplication and assay of viruses.
(b) Cancer research, which requires the study of uncontrolled cell division in cultures.
(c) Cell fusion techniques.
(d) Genetic manipulation, which is easy to carry out in cells or organ cultures.
(e) Production of monoclonal antibodies requires cell lines in culture.
(f) Production of pharmaceutical drugs using cell lines.
(g) Chromosome analysis of cells derived from womb.
(h) Study of the effects of toxins and pollutants using cell lines.
(i) Use of artificial skin.
(j) Study the function of the nerve cells.

(k) Many commercial proteins have been produced by animal cell culture and there medical application is being evaluated. Tissue Plasminogen activator (t-PA) was the first drug that was produced by the mammalian cell culture by using rDNA technology. The recombinant t-PA is safe and effective for dissolving blood clots in patients with heart diseases and thrombotic disorders.

According to applications of mammalian cell culture, it has a big market with about $40 billion per year, so it can be interested for more research.

1.4. Culture Media

The artificial environment created in the laboratory is generally known as media. Media has two main parts: basal media and serum. Basal medium usually consist of carbon and nitrogen source and other ingredients such as organic and inorganic salts, amino acids and vitamins. Typically basal medium needs to other supplements to provide appropriate environment for mammalian cell culture. This supplement totally is called serum and in the past fetal bovine serum (FBS) with 5-20% concentration has been used for animal cell culture in vitro. Unsolvable and unstable nutrients that carry with these sera create growth factors and hormones and unite and counteract toxic molecules. Meanwhile they provide protease inhibitors (PIs) and other important materials and keep safe cells from strain, shear stress and harms. The role of all constituents is not clear. Proteins, peptides, special factors released during platelet aggregation e.g., PDGF, TGF-β, lipids, lipid transport proteins, carbohydrates, micronutrients such as minerals, etc. Cells differentiate and proliferate with supplemental substance that present in the sera. Meanwhile proteases and free radicals protect cells against of toxic agents. Serum also supplies growth factors and nutrients for cell proliferation and differentiation. Besides that, sera can adjust permeability of cell membrane. In fact they act as the transmitter of supplements such as enzymes, lipids, trace element and etc. On the other hand sera have proteins such as albumin and fetuin that they can reduce greatly unnecessary absorption

in the surface of bioreactors. In addition sera effect on physical condition of environment of cell culture like osmolarity because they have many natural buffers. Serum reduces shear stress on culture in bioreactor. It regulates viscosity of media and rate of gas delivery to the cells.

1.5. Problem Statement

In order to produce serum, first all the blood sera gathered and then frozen and eventually clot. This clot centrifuges and residual material separate, remained liquid will be serum. Sear is essential for growing of cell by basal media but all of its components have not been identified yet. Sera consist of more than 100 varied ingredients. Not only each serum has an unknown composition, but also since sera are provided from different areas, therefore a serum has completely different composition from other serum. Many parameters are involved in the growth of cell and some of these parameters have negative effect and others have positive effect. Effects of these parameters on the cell growth depend on type of cell and medium that is considered for cell culture. So composition of serum has a significant role in cell culture. For example an agent as amine oxidases in serum may hinder from growing of cells in cell culture or is demonstrated that diploid cells have different life span in dissimilar sera.

Usually sera have high protein concentration that it can be undesired for cell growth. Meanwhile it can make difficult downstream processes. Also extra proteins may decrease effect of antibodies because these proteins may act as antigen and it is obvious that neutralized antibody will lessen effect of antibodies.

Sera that have been derived from animal usually consist of several septic agents like viruses, prions and mycoplasma. Not only these agents may impact on cell growth, but also they can inhibit from cells growth and hence downstream products that most of

them are for medicinal use convert to dangerous materials. For instance, vaccinate of animal can rise to Bovine Viral Diarrhea Virus (BVDV) in serum and it will effect on product of mammalian cell culture. So definition of a healthy medium in order to use in manufacturing of clinical and biopharmaceutical products is necessary. In fact many laboratories and manufactures need to appropriate replacement for fetal bovine serum rapidly. Otherwise they will require from sanitization process for purification of serum from these infections (Staines, 2003).

Besides these reasons, using of fetal bovine serum creates doubt for Muslims because base of the holy Qur'an, animal blood is unclean and utilizing this blood is banned by Islam. More than one billion Muslim people in the world need Halal Products. Therefore, there could be the need for Halal medium for biopharmaceutical production in future.

Many authors proposed several mediums. Serum-free medium, protein-free medium, animal-derived component-free medium and chemically defined media are types of different medium that have suggested by researchers. Since each cell needs to special defined medium, many researchers have worked on the different cell culture medium for dissimilar cells. These studies involved the finding of appropriate replacement for serum or developing of a medium without serum. Numerous proteins, carbohydrates, amino acids, hormones, enzymes, vitamins, growth factors, lipids and other sources have been tested over the past decade.

This study proposes similar objectives as compared to previous researches. The proposed study seeks to use plant derived protein in culture medium as a supplement. This work could lead to the finding of complete serum replacement from plant in future.

1.6. Objective

The main objective of this work is to use plant protein hydrolysates as a supplement for medium of human skin fibroblast (HSF) 1184 cell culture.

1.7. Scope

1. Protein extraction from plants like Soy, Sesame, Wheat, Rice and *Aloe vera* by different methods and enzymes such as Alcalase, Flavourzyme, Alcalase→Flavourzyme, Papain and Bromelain.

2. Growth profile of Fibroblast 1184 cell culture exposed with different hydrolysed proteins.

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