# THE EFFECT OF ELECTRON BEAM IRRADIATION ON THE QUALITY OF HYDROLYSED COLLAGEN AND CELL VIABILITY

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# ABSTRACT

The purpose of this study was to evaluate the effect of electron beam irradiation at different dosages towards the quality of hydrolysed collagen samples from *Oreochromis mossambicus*'s scales. Hydrolysed collagen samples powder were irradiated using electron beam at doses of 5, 10, 15 and 20 kGy. After being irradiated, samples were individually vacuum-packaged and stored at 4°C for four weeks. Then, the total number of coliform, yeast and mould, pH, macronutrients of samples, and molecular weight of the samples' protein were investigated. This study also determined the effect of hydrolysed collagen samples at different concentrations towards the viability and proliferation of skin fibroblast primary cell. The results showed that total number of coliform, yeast and mould decreased with the increment of irradiation dosage, but, no coliform, yeast and mould growth in 15 and 20 kGy samples. Meanwhile, the $D_{10}$ value of coliform is 14.43 kGy whilst yeast and mould is 13.76 kGy. The samples' pH values and macronutrients evaluation did not show any significant differences between non-irradiated and irradiated samples. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separated two bands of protein between 17.75 to 23.5 kDa. It also showed that electron beam irradiation dosage did not cause any substantial change of electrophoretic pattern on molecular weight of hydrolysed collagen. The concentration of hydrolysed collagen between 4 mg/mL to 10 mg/mL has increased the viability and proliferation of normal skin fibroblast primary cell. As a conclusion, the results suggest that electron beam irradiation is useful in improving microbial safety without impairing the quality of hydrolysed collagen. The implication of this study is hydrolysed collagen from *O.mossambicus*'s scales can be used to increase the number and proliferation of skin fibroblast cell and would be essential for any skin care and pharmaceutical use.

# KESAN IRADIASI SINARAN ELEKTRON TERHADAP KUALITI KOLAGEN TERHIDROLISIS DAN KEUPAYAAN HIDUP SEL

## ABSTRAK

Kajian ini dijalankan untuk menilai kesan iradiasi sinaran elektron pelbagai dos terhadap kualiti kolagen terhidrolisis daripada sisik *Oreochromis mossambicus.* Serbuk kolagen terhidrolisis diiradiasikan pada dos 5, 10, 15 and 20 kGy. Selepas diiradiasi, serbuk tersebut akan dipaketkan secara individu dan disimpan pada suhu 4°C untuk penyimpanan selama empat minggu. Jumlah populasi koliform, yis dan kulat, pH, makronutrien, dan berat molekul sampel protein telah dikaji. Selain daripada itu, kesan kolagen terhidrolisis terhadap sel kulit fibroblas juga dikenal pasti berdasarkan bilangan dan percambahan sel. Keputusan menunjukkan bahawa bilangan populasi koliform, yis dan kulat semakin menurun apabila dos iradiasi bertambah, tetapi, tiada sebarang pertumbuhan koliform, yis dan kulat pada kolagen terhidrolisis yang diiradiasikan pada dos 15 dan 20 kGy. Nilai dos $D_{10}$ bagi perencatan koliform ialah 14.43 kGy manakala nilai dos $D_{10}$ bagi perencatan yis dan kulat ialah 13.76 kGy. Nilai pH dan penilaian makronutrien tidak menunjukkan sebarang perbezaan yang signifikan antara sampel yang telah diiradiasi dengan yang tidak diiradiasi. Elektroforesis gel poliakrilamida sodium dodesil sulfat (SDS-PAGE) telah memisahkan dua jalur, iaitu antara 17.75 hingga 23.5 kDa. Pemisahan jalur ini juga tidak menunjukkan sebarang perubahan ketara antara sampel yang telah diiradiasi dengan yang tidak diiradiasi. Kepekatan kolagen terhidrolisis yang diiradiasi pada dos 15 kGy antara 4 mg/mL hingga 10 mg/mL telah meningkatkan jumlah hidup dan percambahan sel kulit fibroblas. Sebagai kesimpulan, keputusan kajian ini mencadangkan bahawa iradiasi sinaran elektron berguna dalam meningkatkan keselamatan daripada mikrob tanpa merosakkan kualiti kolagen terhidrolisis. Implikasi kajian ini adalah kolagen terhidrolisis daripada sisik *O. mossambicus* dapat digunakan dalam peningkatan bilangan dan percambahan sel fibroblas kulit, di samping sesuai digunakan untuk produk penjagaan kulit dan farmaseutikal.

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| | |
|---|---|
| Ala-Hyp-Gly | Alanine-Hydroxyproline-Glycine |
| ANOVA | Analysis of Variance |
| AOAC | Association Official Analytical Chemist |
| APS | Ammonium Persulfate |
| $a_w$ | Water Activity |
| $cm^2$ | Cubic Centimetre |
| $^{60}Co$ | Cobalt-60 |
| $^{137}Cs$ | Caesium-137 |
| $CuSO_4$ | Cuprum Sulphate |
| $D_{10}$ value | Values which is the Irradiation Dose Required to Reduce Total Coliforms, Yeast and Mould Number by 90% |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EMB | Eosin Methylene Blue Agar |
| FDA | Food and Drug Administration |
| $[J.kg^{-1}]=[Gy]$ | Absorbed Energy per Unit Mass |
| kDa | Kilo Dalton |
| kGy | Kilo Gray |
| $kJ/m^2$ | Kilojoule per Cubic Metre |
| $K_2SO_4$ | Potassium Sulphate |
| log CFU $g^{-1}$ | Logaritm Colony Forming Unit per Gram |

| | |
|---|---|
| M | Molarity |
| mA | Beam Current |
| MeV | Acceleratre Voltage |
| mg/mL | Miligram per Mililitre |
| MPN | Most Probable Number |
| Mrad | Miliradian |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole |
| NaCl | Sodium Chloride |
| NaOH | Sodium Hydroxide |
| nm | Nanometre |
| $OH^-$ | Hydroxyl Group |
| pH | Potential of Hydrogen |
| Pro-Hyp | Proline-Hydroxyproline |
| RGD | Arginine(R) – Glycine(G) – Aspartic Acid(D) |
| rpm | Revolution per Minute |
| SDS PAGE | Sodium Dodecyl Polyacrylamide Gel Electrophoresis |
| SPSS | Statistical Packages for the Social Science |
| TEMED | N,N,N',N' – tetramethylethylenediamine |
| Tris-HCl | Tris-Hydrochloric Acid |
| $T_m$ | Crystalline Melting Temperature |
| UV | Ultra Violet Radiation |
| w/v | Weight per Volume |
| µg/mL | Microgram per Mililitre |


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# CHAPTER 1

# INTRODUCTION



## 1.1 Background of Study

Collagen is the main structural protein in extracellular matrix, acts an important role in maintaining structural requirement of extracellular matrix. It also involves in reconstructing for physiological functions (Pati, Datta, Adhikari, Dhara, & Ghosh, 2012) and plays role in the structure of several tissues, such as skin and bones, providing rigidity and integrity as well as in regenerative processes (Ricard Blum, 2011).

A biomaterial can be recognized as inorganic or organic materials that are biocompatible and suitable to be used in human body in order to substitute or repair damaged tissue. Due to its biodegradability and weak antigenicity, collagen recently

being an important biomaterial in medical applications (Lee, Singla, & Lee, 2001). For these reason, the collagen usage is widely used in pharmaceutical and biomedical industry as surgical sutures, cosmetics, wound healing and tissue engineering (Cen, Liu, Cui, Zhang, & Cao, 2008) either alone or in combination with other biomaterials (Friess, 1998).

When collagen is being hydrolysed, a collagen peptide or also known as collagen hydrolysate is being formed. This hydrolysed collagen is a polypeptide composite made by further hydrolysis of denatured collagen. The hydrolysis involves breaking down molecular bonds between collagen strands using heat, acids or enzymes. It has been used in cosmetic preparations and skin treatment as an effective hydrating agent (Morimura et al., 2002) and potentially in treatment of bone and joints diseases (Bruyère et al., 2012). It has required benefits for many applications as an important biological nutrient because of its harmless effect to human health and water retention capability. Rather than gelatin, hydrolysed collagen has lower molecular weight, thus make it greater in absorption and penetration (Pei et al., 2013).

Normally, type I collagen that is obtained from bovine source such as cow, pig, and cattle is used for medical applications. Collagens of human recombinant recently being produced in mammalian, bacterial, yeast, and plant systems, but their production is very slight and still not used for large scale application (Ramshaw, Werkmeister, & Glattauer, 1996). Various kinds of alternative sources for collagen have been approved such as marine and fresh water fishes, marine animals like squid, octopus, jellyfish, starfish and many others (Nagai et al., 2004). However, recent outbreak of flu that has disease transmission risk which come from pig and other

bovine source has been found. Thus, fish processing wastes is comparatively safer and essential as an alternative in collagen extraction (Pati et al., 2012).

The nature of fish collagen that has low denaturation temperature will result in more sensitive to heat denaturation rather than collagen from bovine source. Thus, fish collagen is difficult to be used as biomaterials (Nagai et al., 2004). Lower stability is caused by the lower hydroxyproline content in fish collagen when comparing with bovine collagen. Hydroxyproline content will affecting thermal stability because of the intramolecular hydrogen bonds between hydroxyl ($OH^-$) groups of hydroxyproline stabilized the triple helix of collagen (Swatschek, Schatton, Kellermann, Müller, & Kreuter, 2002).



To overcome this problem, fish scale collagen from tilapia species was found to be quite heat stable. It has higher thermal stability with higher resistance of heat, and their better structural stability might be useful to replace the mammalian collagen (Huang, Kuo, Wu, & Tsai, 2016). Other than that, another collagen from fish scale (*Rohu* and *Catla* fish) was found to have denaturation temperature which is advantageous for biomedical application, food and cosmetic industries due to closeness in denaturation temperature to mammalian collagen (Pati, Adhikari, & Dhara, 2010).

South East Asia country like Indonesia, Malaysia and Thailand is a tropical country that produces warm water fish species. Many studies have reported that collagen from warm water fish species contains more amino acids rather than the species from cold water fish (Gudmundsson, 2002). Several organizations in Malaysia

have conducted a few research and development on collagen. Malaysia Dairy Industries has used hydrolysed collagen in their nutritional drink. The hydrolysed collagen also served as components required in synthesizing collagen in our skin. Furthermore, vitamin C as a vital coenzyme in collagen biosynthesis was added with hydrolysed collagen. Avon Life Marine Peptide Collagen Drink was also being produced by Avon, which obtained from natural and high quality of fish hydrolysed collagen. Other than that, Kacip Fatimah, Nescafe Body Partner, and Collagen Coffee that was added fish hydrolysed collagen in their ingredients has also released by Nestle Malaysia (Hashim, Mohd Ridzwan, Bakar, & Mat Hashim, 2015).

Hydrolysed collagen consists of small peptides with low molecular weight, enriched in specific amino acids such as glycine, proline, and hydroxyproline (Liu, Liang, Regenstein, & Zhou, 2012). Due to its low molecular weight, hydrolysed collagen is highly digestible, absorbed and distributed in the different tissues of the human body. Several experiments have shown that collagen peptides can be efficiently absorbed and distributed to the dermis, where they can stimulate the proliferation and motility of fibroblast cells (Sibilla, Godfrey, Brewer, Budh-Raja, & Genovese, 2015).

Irradiation is a process for the treatment of food or products to enhance their shelf life and to improve microbial safety. For food and cosmetic products, electron beams at energy levels up to 10 MeV are permitted. Although electron beam irradiation is less penetrating than gamma rays, they can be very useful in reducing the microbial spoilage (Venugopal, Doke, & Thomas, 2014). In addition, UV irradiation could also reduce contamination levels of product because of its broad

antimicrobial action, providing effective inactivation of vegetative bacteria, bacterial spores and yeast (Falguera, Garza, Pagán, Garvín, & Ibarz, 2013).

## 1.2 Problem Statement

Nowadays, extract of collagen from fish skins, scales, and bones have become an alternative source, due to the presence of animal related contamination risk. Compared with collagen, hydrolysed collagen allowed easier direct absorption by the human body. The lower molecular weight of hydrolysed collagen not only goes through the skin's stratum corneum, but also penetrates the deep dermal layer. Other than that, they have the same important role as human peptides in tissue repair and regeneration of skin (Wang, Zhang, Zhang, & Li, 2011).



In particular, several organizations in Malaysia have manufactured collagen and hydrolysed collagen from different sources, and has been widely used in Malaysia itself because of its various functions (Hashim et al., 2015). However, although the moisture content of fish collagen is low (Jamilah, Umi Hartina, Mat Hashim, & Sazili, 2013), there will still be microbial safety problems during marketing and distribution. For therapeutic preparation, any products should meet the microbial safety guidelines during storage and marketing. Generally, microbial contamination of medical products is a problematic, and the conventional methods which is fumigation with gaseous ethylene oxide or methyl bromide, are banned for the sake of health concern (Jin, Shin, & Song, 2007).

The electron beam acecelerator is a relatively new, flexible and more effective source for food and cosmetic irradiation rather than gamma rays. The electron beam is easy to adapt to different radiation process requirements, such as operating at different beam energy levels. No radioactivity is present when the accelerator is off, therefore, no radioactive waste will be accumulated, whereas usually 16 to 21 years are required to dispose of $^{60}$Co (Venugopal et al., 1999).

Electron beam and UV irradiaton are the most common types of irradiation for sterilization purposes (Jin et al., 2007). Gamma ray application for food preservation was not commercially used due to the consumer scepticism regarding the wholesomeness of foods or cosmetics that are irradiated with gamma ray from radioactive isotopes such as $^{137}$Cs and $^{60}$Co. To overcome this problem, application of electron beam and UV to foods or cosmetics without using any radioactive source was introduced (Kim, Chun, Song, & Song, 2010). In addition, electron beam irradiation enhances the thermal stability of fish gelatin and collagen, as well as increasing the glass transition temperature and degradation temperature (Benbettaïeb, Karbowiak, Brachais, & Debeaufort, 2015).

Despite of several microbial studies, quality evaluations are still the most reliable and universally applied to judge the quality of product after being irradiated. Due to the limited study on the effect of electron beam irradiation on qualities of hydrolysed collagen, more research focusing its effect on the qualities of microbiological analysis, pH reading, molecular weight determination, and macronutrients evaluation are needed.

There is a study indicated that gamma ray induced insignificantly change in molecular weight of liquid egg. However, gamma irradiation was found to induce about 1% of molecular aggregation in protein material at a dose as low as 1.5 kGy. In addition, small amount conversion of α-helix to random coil was reported in egg white protein after irradiated with gamma ray at a dosage of 16 kGy. At a contrary, in most experiment, no significant change in amino acid molecular weight when irradiated with electron beam at a dosage below 50 kGy on white and egg yolk (Hong, Ryu, & Kim, 2014). However, studies on electron beam irradiation effects on another protein like collagen and gelatin are very limited and scarce.

When protein materials are irradiated with electron beam treatment, all the reactions that have connection with amino acids are also possible affecting the macromolecules. A large proportion of radiation energy will deposited in an irradiated protein resulting into protein denaturation, although much less when compared with heating. Some protein radicals that are formed by direct action will undergo several futher reactions that will change their macromolecules value (Y. Zhang, Su, Venugopal, Ramakrishna, & Lim, 2007).

The effect of irradiation on physicochemical, macromolecules and nutritional value in the food and cosmetic products depends on a variety of factor such as initial compositon, temperature during irradiation, as well as treatment conditions such as radiation source used, dose rate, and absorbed dose (Hong et al., 2014). The study from Y. Zhang et al., (2007) stated that the changes including loss of vitamins are minimal at lower doses of electron beam irradiation in treated food, and has been agreed that the extent of nutritional losses is comparable less than that of the most

other food processing. However, studies on electron beam irradiation effects towards macromolecules of pharmaceutical or cosmetic product like collagen are very limited.

The application of hydrolysed collagen from many sources will be realized upon immunological response, biocompatibility, tissue engineering, and other safety concern (Pati et al., 2012). However, there are scarcity information regarding the impacts of electron beam irradiation on the qualities of microbiology analysis, molecular weight determination, and macronutrients evaluation. Only a few reported publications on the toxicity of collagen showed that collagen is very beneficial for the growth of cell, especially to fibroblast cell (Wang et al., 2011), but the studies are very specific regarding the sources of collagen. To address the issue, this study was undertaken to establish a profile for various concentration of hydrolysed collagen from *Oreochromis mossambicus's* scale towards the viability and proliferation of normal skin fibroblast primary cell.



## 1.3 Research Objectives

This research aimed to investigate the effect of different electron beam irradiation dosage towards the quality on fish scale hydrolysed collagen and cell viability. In more specific, this study aimed:

1. To determine the efficacy of electron beam and UV irradiation on coliform, yeast and mould inactivation.
2. To determine the pH and macronutrients evaluation changes of hydrolysed collagen after being irradiated during storage.

3. To determine the molecular weight of hydrolysed collagen and its changes after being irradiated.
4. To determine the viability and proliferation of skin fibroblast primary cell towards hydrolysed collagen.

## 1.4 Research Questions

This study was carried out based on research questions as below:

1. Does electron beam sterilization affect the inactivation of coliform, yeast and mould when comparing to UV irradiation?
2. How many dosage of electron beam irradiation treatment that reduces the total coliform and yeast and mould number on hydrolysed collagen?
3. Does electron beam irradiation affect the pH of hydrolysed collagen during storage?
4. Does electron beam irradiation effect the macronutrients evaluation of hydrolysed collagen during storage?
5. What is the molecular weight of hydrolysed collagen and its changes after being irradiated?
6. How many concentration of hydrolysed collagen that is beneficial to the viability of skin fibroblast primary cell?
7. Is there any beneficial changes occur in terms of proliferating the normal skin fibroblast primary cell when treated with hydrolysed collagen?

## 1.5 Significance of Study

Since there was limited study on the effect of irradiation towards hydrolysed collagen, this study is important to increase our present knowledge on the effectiveness of irradiation towards our products mainly food, cosmetics or pharmaceutical. This study will provide the evidence about the effect of irradiation in terms of physicochemical properties and the quality of products. Thus, electron beam irradiation has been selected to be used in this study due to the shorter processing time and does not produce radioactive waste (Jin et al., 2007).

This study is also essential for several organizations in Malaysia that are intended to use hydrolysed collagen in their product. Through the viability and proliferation test of cell towards hydrolysed collagen, this study revealed about the safety of collagen usage in our daily life, especially to the cosmetics collagen users. In addition, it is valuable for improving the concentrations of hydrolysed collagen in a product. Understanding about the quality of hydrolysed collagen after being irradiated, viability and proliferation test of cell, will assist the health ministry in monitoring the new products in our country. Thus, this knowledge application will give positive impacts to hydrolysed collagen yield as well as to our economic income.

## 1.6 Scope and Limitations of Study

This study was carried out within the scopes and limitations as below:

1. The source of *Oreochromis mossambicus*'s scale hydrolysed collagen was obtained from Chemistry Department, Universiti Pendidikan Sultan Idris.
2. The electron beam irradiation dosage was 5kGy, 10kGy, 15kGy and 20kGy.
3. Selected parameters for quality test were microbiological analysis, pH measurement, molecular weight determination and macronutrients evaluation.
4. Selected parameters for cell viability test were MTT assay and trypan blue assay.
5. The cell viability and proliferation test was specific towards the normal skin fibroblast primary cell.
6. The concentration of fish scale hydrolysed collagen that treated the normal skin fibroblast primary cell was ranged between 0.1-10 mg/mL (0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, 10).
7. The microscope used for cell proliferation observation was inverted microscope.

Thus, the result of this study is only valid for all the parameters, situations, and materials as mentioned above. It cannot be generalized to all types of hydrolysed collagen.

## 1.7 Research Design and Hypothesis

This study was divided into two parts: (i) quality on fish scale hydrolysed collagen and (ii) cell viability and proliferation towards fish scale hydrolysed collagen.

First Part: Quality on fish scale hydrolysed collagen.

The quality test of non-irradiated and irradiated hydrolysed collagen includes microbiological analysis, pH measurement, macronutrients evaluation and molecular weight determination. Extracted hydrolysed collagen was obtained from Chemistry Department, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris and sent for electron beam irradiation at Malaysia Nuclear Agency. This part of study was conducted based on the following hypothesis:



(a) Increasing the dosage of electron beam irradiation treatment will reduce the total coliform and yeast and mould number on fish scale hydrolysed collagen.

(b) The $D_{10}$ values which is the irradiation dose required to reduce total coliforms and yeast and mould number by 90% were between 10 kGy to 15 kGy.

(c) Effect of irradiation treatment on total coliform and yeast and mould number was sustained during four weeks storage.

(d) The effect of electron beam irradiation in terms of pH measurement and macronutrients evaluation is not significantly different between non-irradiated and irradiated hydrolysed collagen.

(e) The electron beam irradiation treatment does not alter the protein band number and electrophoretic patterns.

Second Part: Viability and proliferation of fibroblast skin primary cell towards fish scale hydrolysed collagen.

The $D_{10}$ value which is the irradiation dose required to reduce total coliforms and yeast and mold number by 90% was chosen and used for the viability of cell test. The viability of cell include MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay, trypan blue assay and morphological changes of cell. This part of study was conducted based on the following hypothesis:

(f) Increasing the concentration of hydrolysed collagen will increase the optical density reading of normal skin fibroblast primary cell for MTT assay.

(g) Increasing the concentration of hydrolysed collagen will increase the total number of viable normal skin fibroblast primary cell for trypan blue assay.

(h) Beneficial changes occur in terms of proliferating the normal skin fibroblast primary cell when increasing the concentration of hydrolysed collagen.

Table 1.1

*Simplified research design*

| Part | Objective | Parameter | Methodology | Data Presentation / Analysis |
|---|---|---|---|---|
| I | 1 | - Microbial counts of coliform and yeast and mould during 4 weeks storage<br>- The $D_{10}$ –values | - Visual examination of possible coliform and yeast and mould<br>- Microbial counts were expressed as log CFU/g | -Photos<br>-Column chart, Linear regression ($D_{10}$ –values)<br>-One way ANOVA and repeated measure |
| | 2, 3 ,4 | - pH measurement<br>- Macronutrients evaluation<br>- Molecular weight determination | - Analysed according to British Standard Method BS 757<br>- Analysed according to Association Official Analytical Chemist (AOAC 2000)<br>- Analysed according to Laemmli method | - Table,<br>- One way ANOVA and repeated measure<br>- Band photos detected by using GS-800 Calibrated Densitometer |
| II | 5, 6 | - Optical density reading (MTT assay)<br>- Total number of viable cells (Trypan blue assay)<br>- Cell proliferation | -Visual observation by using inverted microscope, cell counting by using haemocytometer, optical density by using microplate reader at 570 nm wavelength | - Column chart with mean ± standard deviation, one way ANOVA and repeated measure, diagram of cell captured from inverted microscope |