UNIVERSITI PUTRA MALAYSIA

IN VITRO IRON-BINDING AND IRON UPTAKE CAPACITIES OF DEFATTED RICE BRAN PEPTIDE HYDROLYSATE

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IN VITRO IRON-BINDING AND IRON UPTAKE CAPACITIES OF DEFATTED RICE BRAN PEPTIDE HYDROLYSATE

By

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***IN VITRO* IRON-BINDING AND IRON UPTAKE CAPACITIES OF DEFATTED RICE BRAN PEPTIDE HYDROLYSATE**

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January 2016

Chair : Maznah Ismail, PhD
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Iron deficiency anaemia (IDA) is a global public health problem affecting 24.8% of the world population with over 90% coming from developing countries. In Malaysia, a prevalence of 35% anaemia has been reported. Iron deficiency has been associated with serious physiological consequences as well as deleterious effects on economies. To overcome IDA, iron fortification in food was used as an alternative approach to improve iron bioavailability. In the present study, the functional properties of defatted rice bran (DRB) peptides were explored as potential iron-binding peptides to enhance iron uptake in human.

The DRB is a by-product of oil extraction from rice bran by supercritical fluid extraction (SFE). SFE is a green technology and yield-promising extraction technique which has shown superiority in many aspects over traditional extraction methods including Soxhlet extraction. The present study showed that defatting of rice bran by SFE yielded similar protein contents as compared with other extraction methods; the maximum amount of protein was obtained over mild SFE conditions (450 bars, 90 min of extraction, and 17.5 g/min flow rate). The study revealed that SFE could be used for the extraction of oil from rice bran while conserving the protein content in defatted residues. Besides that, amino acid analysis of DRB showed that the amino acids in DRB were in sufficient quantity required for an adult (except methionine and cysteine) and met the amino acid score based on the reference protein of FAO/WHO/UHU.

Bioactive peptides from DRB after treatment with food-based proteolytic enzymes exert better biological functions or physiological effects on the peptide itself. Therefore, the iron binding capacity of the DRB protein hydrolysates as affected by enzyme types and degree of hydrolysis (DH) were also studied. DRB hydrolysates produced by combination treatment of an endo-protease (Alcalase) and exo- protease (Flavourzyme) were found to have the best iron-binding capacity (83%) at the 90 min time of hydrolysis. Furthermore, iron cell retention, transport, and uptake from DRB peptide hydrolysates after digestion were investigated using *in vitro* digestion/Caco-2 cells model. Transport and uptake efficiency of DRB hydrolysates obtained using combined treatment of Alcalase and Flavourzyme were significantly higher than those of other DRB hydrolysates and DRB protein without enzymatic hydrolysis. The optimal hydrolysis time to produce the best iron-binding DRB hydrolysate using combination treatment of Alcalase and Flavourzyme was 180 min. Overall, the results support the

application of iron-binding peptides from DRB processing by-products in food fortification product.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

IN VITRO KEUPAYAAN PENGIKAT-BESI DAN PENGAMBILAN BESI DALAM PEPTIDA HIDROLISAT DARIPADA DEDAK PADI YANG DINYAH LEMAK

Oleh

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Pengerusi : Maznah Ismail, PhD
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Anemia akibat kekurangan zat besi (IDA) adalah masalah kesihatan awam global yang mempengaruhi 24.8 % penduduk di seluruh dunia dengan lebih 90 % datang dari negara-negara membangun. Di Malaysia, 35 % penyakit anemia telah dilaporkan. Kekurangan zat besi telah dikaitkan dengan kesan-kesan fisiologi yang serius dan membawa kesan negative terhadap ekonomi. Untuk mengatasi gejala IDA, penambahan zat besi dalam makanan telah digunakan sebagai pendekatan alternatif untuk meningkatkan bioavailabiliti besi. Dalam kajian ini, sifat-sifat dan fungsi peptida dedak padi yang dinyah lemak (DRB) telah diterokai sebagai peptida besi-terikat yang berpotensi untuk meningkatkan keberkesanan serapan zat besi dalam manusia.

DRB adalah sisa produk daripada pengekstrakan minyak daripada dedak padi oleh pengekstrak cecair CO_2 (SFE). SFE ialah teknologi hijau yang menjanjikan hasil pengekstrakan yang lebih berkesan. Ia telah menunjukkan keunggulan dalam pelbagai aspek berbanding daripada kaedah pengekstrakan konversional termasuk pengekstrakan Soxhlet. Kajian ini menunjukkan bahawa dedak padi yang dinyah lemak oleh SFE menghasilkan kandungan protein yang sama berbanding dengan kaedah pengekstrakan yang lain; jumlah maksima protein telah diperolehi dengan parameter SFE yang sederhana (450 bar, 90 min pengekstrakan, dan 17.5 g kadar aliran/min). Kajian ini mendapati SFE sesuai digunakan untuk pengekstrakan minyak daripada dedak padi sambil mengekalkan kandungan protein dalam sisa yg dinyah lemak. Selain itu, analisis kandungan amino asid DRB menunjukkan bahawa kuantiti amino asid dalam DRB adalah mencukupi pada kadar yang diperlukan untuk orang dewasa (kecuali methionine dan cysteine) dan menemui skor kandungan amino asid protein berdasarkan rujukan daripada FAO / WHO / UHU.

Peptida bioaktif daripada DRB selepas rawatan dengan enzim proteolitik yang berasaskan makanan, fungsi biologi atau kesan fisiologi yang lebih baik daripada peptida asal. Oleh itu, keupayaan pengikat besi DRB hidrolisat protein juga dikaji dengan mempelbagaikan perlakuan jenis enzim dan tahap hidrolisis (DH) yang berbeza. Hidrolisat DRB dihasilkan oleh rawatan kombinasi endo-protease (Alcalase) dan ekso-protease (Flavourzyme) didapati mempunyai kapasiti pengikat-besi yang terbaik (83 %) pada masa hidrolisis 90 min. Selain itu, penyimpanan, pengangkutan, dan pengambilan zat besi dari hidrolisat peptida DRB dalam sel hidup telah dikaji dengan menggunakan

penghadaman/*in vitro* model sel Caco-2. Pengangkutan dan kecekapan pengambilan hidrolisat DRB diperolehi dengan menggunakan rawatan gabungan Alcalase dan Flavourzyme adalah lebih tinggi berbanding dengan hidrolisat DRB yang lain dan juga protein DRB tanpa hidrolisis enzim. Masa hidrolisis optimum untuk menghasilkan besi terikat terbaik DRB hidrolisat menggunakan rawatan kombinasi Alcalase dan Flavourzyme adalah 180 min. Keseluruhannya, kajian ini menyokong kegunaan peptida pengikat-besi dari pemprosesan DRB - produk sebagai produk makanan yang diperkayakan zat besi.

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I certify that a Thesis Examination Committee has met on 13 January 2016 to conduct the final examination of Foong Lian Chee on her thesis entitled "*In Vitro* Iron-Binding and Iron Uptake Capacities of Defatted Rice Bran Peptide Hydrolysate" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
Asp	Aspartic acid
ATP	Adenosine triphosphate
Au g^{-1}	Arbitrary unit(s) per gram
BERNAS	Padiberas Nasional Berhad
CPP	Casein phosphopeptides
Co.	Corporation
DMSO	Dimethyl sulfoxide
DH	Degree of hydrolysis
DMEM	[illegible]
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DRB	Defatted rice bran
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration, US
$FeSO_4$	Ferrous sulfate
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HPLC	High performance liquid chromatography
ID	Iron deficiency
IDA	Iron deficiency anaemia
IRRI	The International Rice Research Institute
kDa	A unit of molecular mass equal to 1000 dalton
Leu	Leucine
Lys	Lysine
MARDI	Malaysian Agricultural Research and Development Institute
MPa	Megapascal, a metric pressure unit and equals to 1 000 000 force of newton per square meter which is a pascal.
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
PBS	Phosphate buffered saline
Phe	Phenylalanine
PITC	Phenyl isothiocyanate
Pro	Proline
RB	Rice bran
RDA	Recommended daily amount
Ser	Serine
SDS	Sodium dodecylsulphate
SFE	Supercritical fluid extraction
SH	Sulfhydryl
TEA	Triethylamine
TEER	Transepithelial Electrical Resistance
Thr	Threonine
Tyr	Tyrosine
U/ml	Unit enzyme per milliliter

WHO	World Health Organization
WPC	Whey protein concentrate

CHAPTER 1

INTRODUCTION

1.1 Background

Iron is one of the essential minerals in human nutrition, used in the biosynthesis of cytochromes, haemoglobin, myoglobin and some enzymes (Mustafa, 2011). Insufficient dietary intake of iron is one of the main reasons attributed to iron deficiency (Martínez-Navarrete, Camacho, Martínez-Lahuerta, Martínez-Monzó, & Fito, 2002). Oral iron supplementation is the first line of treatment and prevention for the iron deficiency anaemia. The most commonly used source of supplemental iron, iron sulphate, is relatively inexpensive and in a form readily available to the human. However, low bioavailability and side effects such as nausea, abdominal cramps and constipation have hindered its application (Wang et al., 2011).

Iron fortification in many foods has been considered as a potential approach to prevent iron deficiency in humans. Some food-based proteins and peptides have been reported to possess iron-binding properties including shrimp processing by-product protein hydrolysate, whey protein concentrate hydrolysates, yak casein hydrolysate, and soy protein isolate hydrolysate (Huang, Ren, & Jiang, 2011; Zhang, Huang, & Jiang, 2011; Lee & Song, 2009; Kim et al., 2007; Vattem & Mahoney, 2005; Yeung, Glahn, & Miller, 2002). To provide new opportunities for iron fortification of food, the application of defatted rice bran (DRB) protein hydrolysates was considered a suitable alternative.

Rice bran (RB) is the by-product of rice milling process and constitutes about 10% of the rough rice. RB has been recognised for some time now as a valuable commodity for its oil. Its protein content after the extraction of oil is also of high quality, with a good balance of the essential amino acids. After treatment with food-based proteolytic enzymes, the bioactive peptides exert different biological functions or physiological effects including antioxidative, hypoallergenic, and some anti-cancer effects (Fabian & Ju, 2011; Revilla et al., 2009).

Recently, supercritical fluid extraction (SFE) has gained momentum as a clean, green, non-flammable and a yield-promising extraction technique, which has shown superiority in many aspects over traditional extraction methods including Soxhlet. SFE has been successfully used for the extraction and separation of many bioactive compounds, including gamma-oryzanol from rice bran oil (Ismail, Al-Naqeeb, Mamat, & Ahmad, 2010), thyme extracts from thyme leaves (García-Risco, Vicente, Reglero, & Fornari, 2011), and thymoquinone-rich fraction from *Nigella sativa* (Ismail, Al-Naqeep, & Chan, 2010). The parameters of SFE, i.e. temperature, pressure, flow rate, and extraction time, vary over an arbitrary range and are required to be optimized to improve extraction efficiency. All these optimizations are done in the interest of SFE primary extract. The properties of SFE residues, which are of secondary importance after the separation of primary extracts, has not received proportional focus from researchers.

As properties of primary SFE extract are reported to be strongly dependent on optimization of parameters, the same impact may be expected on the secondary extract, i.e. residue of the DRB. The defatted residues of rice bran obtained from SFE contain heavy amounts of protein (12-18%). Proteins are fragile compounds, where many factors such as pressure and time may cause changes in their structure. Therefore, this study was carried out to study the effect of defatting process on protein content of DRB. For the purpose, protein content in DRB samples obtained at different sets of SFE operational parameters was determined. Efforts were made to correlate the effects of parameters with the yield of proteins. Furthermore, residual oil content and moisture content from DRB samples were determined and correlated with the yield of protein, while effects of enzyme types and degree of hydrolysis on DRB protein hydrolysates were determined. Using an *in vitro* system (*in vitro* digestion/Caco-2 cells model), iron cell retention, transport, and uptake from DRB protein hydrolysates after digestion were equally investigated.

1.2 Problem statement

Iron-deficiency anaemia is the top ranking cause of anaemia in the world (Kassebaum et al., 2014), affecting billions of people especially in the region of South-East Asia (McLean, Cogswell, Egli, Wojdyla, & de Benoist, 2009). In Malaysia, anaemia is a major public health problem (Ngui, Lim, Chong Kin, Sek Chuen, & Jaffar, 2012; Haniff et al., 2007; Foo, Khor, Tee, & Prabakaran, 2004), with a prevalence of 35% anaemia among pregnant mothers attending the public antenatal clinics of the 14 states in Malaysia (Haniff et al., 2007).

Iron deficiency affects bodily functions severely and especially causes tiredness, GLIIILFXOW\ FRQFHQWUDWLQJ DV ZHOO DV LPSDLUPHQW development (Kassebaum et al., 2014; 0 D UWLQH] Navarrete et al., 2002; Beard, Dawson, & Pinero, 1996). In addition to the deleterious effects of iron deficiency on physiological systems in individuals, the resulting public health consequences can significantly affect economies in terms of public health costs, wasted educational resources, restraint of productivity, and loss of human capital formation (World Health Organisation, 2001).

Although some iron fortified foods have been reported, the practise and popularity of iron fortification in foods is still a challenge in Malaysia. Highly abundant of the iron fortified food source, increased bioavailability of the iron and decreased cost in food sources would justify their purchase and inclusion in human diets.

1.3 Significance of the study

The use of DRB protein and hydrolysates in this study is of particular interest due to their availability and low costs due to the efficient utilization and value-addition of the waste products, as well as being an environment friendly alternative. In Malaysia, rice is an important crop, producing up to 1.75 million tonnes of rice in 2014 (FAO Rice Market Monitor, 2014). If the RB from this much rice were to be discarded as waste, it would become apparent how much is lost in terms of health-promoting food. This ³ZDVWHH DOWK´ FRQFHSW LV WKH EDVLV EHKLQG WKH K potential source of functional ingredients.

Moreover, RB has high quality protein due to its essential amino acid. Its nutritional and bioactive compositions of DRB also it can be argued that it may have benefits in a wide range of applications including the use of its protein and hydrolysates for iron-binding. It was hypothesized that DRB protein obtained after extraction of RB oil using SFE could be a good iron-binding source and that its protein hydrolysates could be incorporated as a functional ingredient in iron- I R U W L ¿ H G V X S S O H P H Q W V

1.4 Objective of Research

General Objective

To investigate the *in vitro* iron binding and iron uptake capacities of defatted rice bran (DRB) peptide hydrolysates.

Specific Objectives

1. To determine the effect of Supercritical Fluid Extraction (SFE) parameters on protein, moisture, and residual oil contents in DRB obtained after extraction of oil to explore potential of its active constituents.
2. To characterise DRB protein concentrate and enzymatically-treated DRB protein hydrolysates in relative to their iron binding activity as affected by different enzymes and degree of hydrolysis.
3. To determine the effect of DRB protein hydrolysates in enhancing iron absorption using the combined model of *in vitro* digestion and Caco-2 cells.

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