UNIVERSITI PUTRA MALAYSIA

COCOA-SPECIFIC AROMA POTENTIAL OF SELECTED SEEDS GLOBULIN BY IN VITRO PROTEOLYSIS WITH COCOA PROTEASES

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by

RASHIDAH BINTI SUKOR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Specially dedicated to my beloved husband and soulmate,

Ja'afar As-Sidek bin Mohd. Sidek

for the unconditional love, patience, support and understanding.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Masters of Science

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This study was carried out to study the globulin characteristics from cottonseed, alfalfa seed, pea, mung bean and French bean and compare their characteristics to cocoa seeds. The study also examined whether these globulins from selected seeds are capable in producing cocoa-specific aroma precursors through proteolysis with cocoa proteases. The isolated globulins were characterized for molecular weight by SDS PAGE, amino acid and oligopeptide profile by High Pressure Liquid Chromatography (HPLC). The globulins were initially treated with an endoprotease for 16 h at 50°C, pH 5.2 and subsequently with a carboxypeptidase for another 16 h at 45°C, pH 5.8; both crude enzymes were extracted from cocoa acetone dry powder (AcDP). Proteolysis products were roasted at 120°C for 20 min with reducing sugars and deodorized cocoa butter. Sensory evaluation session was conducted to detect distinctive cocoa aroma in the proteolysis products. The aroma compounds were extracted through Steam Distillation Extraction (SDE) and analysed using Gas Chromatography (GC).

Alfalfa seed gave the highest total protein of 0.28 mg/mg followed by cottonseed, mung bean, pea, French bean and cocoa with 0.26, 0.25, 0.24, 0.19 and 0.12 mg/mg, respectively. A very low globulin yield was obtained from different seeds, between 0.55% and 2.72%. The seeds had high percentage of crude protein between 13.79 and 26.63%. Two distinctive bands of 51.1 and 33.0 kDa were observed for cocoa vicilin-class globulin (VCG) from SDS PAGE. More than three bands were shown for other seed globulins. Comparative HPLC analyses of the obtained peptide mixtures revealed different and complex patterns of predominantly hydrophobic peptides. After proteolysis, the peptide patterns showed reduced number of peaks, which indicated that peptides have transformed to be more hydrophilic. Considerable differences were observed between the patterns of free amino acids; preferential liberations of hydrophobic amino acids of Ala, Leu, Phe, Val and Tyr were observed in all seeds globulins after proteolysis. No cocoa-specific aroma was detected from the proteolysis products of seeds globulin from cotton, alfalfa, pea, mung bean and French bean. However, pyrazines concentration varied in the proteolysis products derived from those seeds globulin.

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

POTENSI PENGHASILAN AROMA SPESIFIK KOKO OLEH GLOBULIN DARIPADA BIJI BENIH TERPILIH MELALUI PROTEOLISIS *IN VITRO* DENGAN ENZIM PROTEASE KOKO

Oleh

RASHIDAH BINTI SUKOR

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Kajian ini dijalankan untuk mengasingkan globulin yang dihasilkan daripada biji kapas, biji alfalfa, kacang pis, kacang hijau dan biji kacang buncis serta membandingkan ciri-ciri globulin tersebut dengan biji koko dan mengkaji sama ada protin tersebut mampu menghasilkan pelopor aroma spesifik koko. Pencirian globulin yang telah diasingkan dijalankan untuk penentuan berat molekul menggunakan SDS PAGE, profil amino acid dan oligopeptida melalui Kromatografi Cecair Tekanan Tinggi. Globulin-globulin tersebut didegradasi oleh enzim endoprotease selama 16 jam pada suhu 50°C, pH 5.2 kemudiannya oleh enzim carboxypeptidase selama 16 jam lagi pada suhu 45°C, pH 5.8; kedua-dua enzim diekstrak daripada AcDP biji koko. Hasil proteolisis dipanggang pada suhu 120°C selama 20 min dengan gula penurun dan mentega koko yang telah dinyahbau. Latihan sensori dijalankan untuk mengesan kehadiran aroma spesifik koko. Sebatian aroma kemudian diekstrak dengan kaedah Penyulingan Stim dan dianalisis menerusi Kromatografi Gas.

Biji alfalfa memberikan nilai protin keseluruhan yang tertinggi iaitu 0.28 mg/mg, diikuti oleh biji kapas, kacang hijau, kacang pis, biji kacang buncis dan AcDP koko dengan 0.26, 0.25, 0.24, 0.19 dan 0.12 mg/mg masing-masing. Hasil yang amat rendah diperolehi semasa ekstraksi globulin daripada biji-biji tersebut iaitu di antara 0.55% hingga 2.72%. Biji-biji yang dipilih juga memberikan peratus protin kasar yang tinggi di antara 13.79 dan 26.63%. Terdapat dua jalur polipeptida yang jelas pada SDS PAGE untuk globulin kelas vicilin (VCG) biji koko dengan berat molekul 51.1 dan 33.0 kDa. Terdapat lebih daripada tiga jalur pada globulin daripada biji-biji yang lain. Analisis oligopeptida menunjukkan corak yang kompleks dan berbeza yang kebanyakannya hidrofobik. Pengurangan puncak dalam corak oligopeptida diperhatikan selepas proteolisis yang kebanyakannya ditukarkan kepada peptida hidrofilik. Perbezaan yang ketara dapat dilihat pada corak dan nilai asid amino bebas bagi biji-biji yang dibandingkan sebelum dan selepas proteolisis. Pembebasan asid amino bersifat hidrofobik iaitu Ala, Leu, Phe, Val dan Tyr dapat diperhatikan di semua globulin biji benih selepas proteolisis. Tiada aroma spesifik koko dikesan apabila hasil proteolisis globulin dari biji kapas, biji alfalfa, kacang pis, kacang hijau dan biji kacang buncis. Walau bagaimanapun, keputusan penentuan pirazin daripada hasil proteolisis biji benih tersebut didapati berbeza-beza di antara satu sama lain.

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LIST OF ABBREVIATIONS

AcDP	Acetone dry powder
b.p.	Boiling point
DW	Dry weight
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
kDa	Kilo dalton
MBP	Maltose binding protein
MW	Molecular weight
PBC	Perang Besar Clone
PMSF	Phenylmethyl sulfonyl fluoride
PVPP	Polivinyl pyrolidone
rpm	Revolution per minute
S	Sedimentation co-efficient
SD	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
VCG	Vicilin-class globulin
w/w	Weight/weight
w/v	Weight/volume
v/v	Volume/volume

CHAPTER 1

GENERAL INTRODUCTION

Cocoa is an extremely valuable crop, providing the food industry with chocolate, cocoa and cocoa butter, which is used in a variety of food applicants. Much attention has been focused on the unique properties of cocoa butter which comprises about 50% of the beans dry weight, but relatively little attempt has been made to study the bean protein, although these are the second major constituents at 15-20% dry weight.

Malaysia Cocoa Board (1998) reported a drop of about 7% cocoa production level from last year and lower production forecast. Cocoa Malaysia is also facing the competition from other cocoa production countries e.g. Ghana due to their high quality flavour. The substitution of cocoa aroma from other sources is therefore necessary. The globulin from legume seeds may be added to enhance cocoa flavour if their globulin can produce cocoa aroma precursors through proteolysis.

Cocoa fruit is the widely used tropical crop for the manufacture of chocolate and other confectionary products. Cocoa bean will provide a unique aroma after undergoing proper pulp conditioning, sequential fermentation, drying and roasting processes. Fermentation steps produces flavour precursors when

enzymatic reaction occurs. Thus, the flavour and aroma typical of chocolate will be produced during roasting in the manufacture of chocolate.

The detection of the reaction of amino acids and reducing sugars (Rohan, 1963; 1964) and the contribution of the peptides in Maillard reactions (Mohr *et al.*, 1976) are important findings in researches related to the formation of cocoa aroma. These precursors are important in the formation of cocoa aroma during roasting. Biehl *et al.* (1985) has shown that vacuolar storage protein is a source of specific-cocoa aroma precursors which are released from vicilin (7S)-class globulin (VCG) by the action of cocoa proteases (Biehl *et al.*, 1991; Voigt *et al.*, 1994a).

The protein of a variety of plant is degraded on germination, and serves as the source of nitrogen for the various new nitrogen compounds synthesized by the developing seedling and is called storage protein (Derbyshire *et al.*, 1976). On the basis of their acid amino sequences, subunit compositions, and the processing of the corresponding polypeptide precursors, the globular storage proteins of plant studied so far can be assigned into two different classes: the legumin-like (11-12S) globulins and the vicilin-like (7S) globulins (Derbyshire *et al.*, 1976; Higgins, 1984; Muntz *et al.*, 1985; Borroto and Dure, 1987; Shotwell and Larkins, 1988).

Cocoa seeds contain 2 major storage proteins: a 19 kDa albumin which is related to the soy bean trypsin inhibitor (Kunitz,1947), family of protease inhibitor (Spencer and Hodge, 1991; Tai *et al.*, 1991) and vicilin-class globulin (Spencer and Hodge, 1991, 1992; Tai *et al.*, 1991; McHenry and Fritz, 1992; Voigt *et al.*, 1993b). Cocoa-specific aroma precursors were obtained when isolated vicilin-class globulin was successively degraded by the aspartic endoprotease and carboxypeptidase partially purified from ungerminated cocoa seeds. No typical aroma precursors were obtained when the albumin fraction was subjected to proteolysis by aspartic endoprotease and carboxypeptidase from cocoa seeds. Therefore, it has been clearly shown that the cocoa-specific aroma precursor are only derived from vicilin-class globulin of the globular storage protein from cocoa seeds (Biehl *et al.*, 1982a; Voigt *et al.*, 1993a).

The acid amino sequences of the globulins from the coconut (vicilin-class globulin), hazelnuts (legumin class 11-12S), and sunflower seeds (legumin class 11-12S) are rather different with the particular chemical structure of the globulin present in the cocoa seeds, cocoa-specific aroma precursors. Therefore, there was no cocoa or chocolate aroma was generated upon roasting of the proteolysis products (Voigt *et al.*, 1994a).

Statement of Problem

Cocoa aroma is one of the most important characteristics of cocoa flavour. Biehl and Voigt (1994; 1996), indicated that cocoa-specific aroma is produced during roasting of the specific hydrophilic oligopeptides and hydrophobic free amino acids. The specificity depends on the primary structure of VCG of cocoa cotyledons and the splitting types of cocoa cotyledon aspartic endoprotease and carboxypeptidase enzymes. Additionally, the production of these specific components of precursors strictly depends on the cotyledon-pH during fermentation which is controlled by microbial pulp degradation. Thus, the specificity of cocoa aroma depends on the primary structure of cocoa cotyledon VCG, the splitting specificity of cocoa cotyledons proteases and environmental factors.

Neither cocoa albumin nor 7S globulin from hazelnut, sunflower and coconut so far investigated are suitable for producing specific-cocoa aroma precursors by proteolysis (Voigt *et al.*, 1994d). The amino acid sequence of the 65 kDa precursor for the cocoa globulin deduced from the nucleotide sequence of the cloned cDNA revealed considerable sequence homologies with vicilin-class globulin of some dicotyledonous plants, especially with an α-globulin of cotton seeds (McHenry & Fritz, 1992; Spencer & Hodge, 1992). Therefore, this study is being carried out to investigate whether or not cocoa-specific aroma precursors can be derived from globular storage proteins from selected plant

seeds which contains 7S globulin by successive degradation with the aspartic endoprotease and the carboxypeptidase from unfermented cocoa seeds. Seeds of some plants which contain exclusively of 7S globulin have been selected to undergo the proteolysis process.

Objectives of the Study

The objectives of this study were as follows:

1. To isolate and compare the protein characteristics of globulins from cocoa seed to cotton, alfalfa , pea , mung bean and French bean seeds.
2. To study the properties of proteolysis product of cocoa and other seeds globulin after incubation with cocoa endoprotease and carboxypeptidase.

CHAPTER 2

LITERATURE REVIEW

Storage Protein of Legume Seeds

Storage protein of seed plans are utilized as sources of nitrogen, carbon and sulphur, and are accumulated during germination and ripening stages. These elements are synthesized in large quantities during bean maturation in rough endoplasmic reticulum and deposited in the membrane-bound vacuoles, called protein bodies (Baunmgartner & Chrispeel, 1977; Pernollet, 1978; Stegemann, 1975; Graham & Gunning, 1970; Varner & Schidlovsky, 1963). The protein bodies are metabolized again during germination. On average, the percentage of protein in legume seed is 20-25% of dry matter (Derbyshire *et al.*, 1975). Seeds, particularly legume seeds are a high protein food source for man and animals either directly and more recently, as 'textured vegetable protein foods' for man. The functional and chemical properties of seed protein are complex (William *et al.*, 1983)

Derbyshire (1975) reported that many proteins occur in seeds and the problem is to distinguish the storage protein from those, which have metabolic or structural role. Two of the major classes of storage proteins in legume seed are termed vicilin-type-globulin (7S) and legumin-type-globulin (11S) based on their

sedimentation coefficients (Hilda and Octavio, 1995). The widespread occurrence of 11S and 7S type storage globulin in angiosperm seeds has been recognized and accepted (Wolf, 1980).

During their development, plant seeds accumulate large amounts of storage proteins that serve as sources of nitrogen, sulphur and carbon compounds during seed germination (Shotwell & Larkins, 1988). Storage proteins accumulate in membrane-delimited organelles, the protein bodies (Briarty *et al.*, 1970; Pernollet, 1978). Seed proteins can be classified on an operational basis into four solubility classes; albumin (water-soluble), globulin (salt-soluble), prolamin (alcohol-soluble) and glutenin (soluble in dilute acids or alkali) (Osborne, 1924). Plant seeds of different taxa contain rather different proportions of albumin, globulins, prolamins and glutenins (Higgins, 1984; Bewley & Black, 1985; Shotwell & Larkins, 1988). In most monocotyledonous seeds studied so far, prolamins are the major seeds proteins (Bewley & Black, 1985) and in gymnosperms (Misra & Green, 1990) globulins predominate. On the basis of their acid amino sequences, subunit composition, and the processing of the corresponding polypeptide precursors, the globulins studied so far can be assigned to two different classes: the legumin-like and the vicilin-like globulins (Derbyshire *et al.*,1976; Higgins, 1984; Shotwell & Larkins, 1988).