UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERISATION OF COCOA PROTEASE

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By

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Dedicated to
the Body of Christ,
whose members had played
an influential role
in enriching
my life in God.

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BSA	bovine serum albumin
CM	carboxymethyl
DEAE	diethyl aminoethyl
DFP	diisopropyl fluorophosphate
DTT	D,L-dithiothreitol
EDTA	ethylenediamine tetraacetic acid
K_m	Michaelis constant
MARDI	Malaysian Agricultural Research and Development Institute
PCMB	*p*-chloromercuric benzoate
PMSF	phenylmethyl sulphonylfluoride
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SP	sulphopropyl
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
V_{max}	maximal velocity

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science.

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Supervisor : Jinap Selamat, Ph.D.

Faculty : Food Science and Biotechnology

This project was initiated with the intention of isolating and characterising protease from cocoa beans, *Theobroma cacao* Linneaus because a greater knowledge of the proteases present in cocoa beans would lead to a better understanding of the problem of inferior cocoa flavour in Malaysian cocoa beans. The ammonium sulphate fractional precipitation method was used to isolate the cocoa protease while the partial purification of the enzyme was achieved by gel filtration through Sephadex G-200. Four fractions precipitated with 0 - 20%, 20 - 40%, 40 - 60% and 60 - 80% saturations of ammonium sulphate were found to be proteolytically active against casein. Further studies were conducted on the fractions precipitated with 0 - 20% and 20 - 40% saturations of ammonium sulphate. Studies on the isolation procedure showed that the addition of sodium dodecyl sulphate (SDS) or Triton X-100 detergents did not enhance the efficiency

of the isolation process. Temperature studies showed that both the 0 - 20% and 20 - 40% fractions have temperature optima of 45 - 50 °C but were unstable at those temperatures. Both fractions possess more than one pH optima against both casein and bovine serum albumin (BSA). The pH optima are in the strong acidic pH and strong alkaline pH ranges. Inhibitor studies showed that both the 0 - 20% and 20 - 40% fractions are likely to contain cysteine proteases while not ruling out the presence of aspartic proteases. The passage of the 0 - 20% fraction through Sephadex G-200 produced two proteolytically active protein peaks designated as P1 and P2 while that of the 20 - 40% fraction produced five activity peaks which were not well separated. Studies with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the 0 - 20% fraction was relatively pure and that the P1 peak was likely to be a protein aggregate. The characteristics of the proteases in both fractions strongly indicate that these enzymes do play a role in the production of cocoa flavour and its precursors during cocoa fermentation.

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PEMENCILAN DAN PENCIRIAN PROTEASE KOKO

Oleh

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Projek ini dimulakan dengan tujuan untuk memencilkan dan mencirikan protease dari biji koko, *Theobroma cacao* Linneaus, kerana pengetahuan yang lebih mendalam mengenai protease yang hadir dalam biji koko akan memberi kefahaman yang lebih jelas mengenai masalah kelemahan perisa koko dalam biji koko Malaysia. Kaedah pemendakan pecahan amonium sulfat telah digunakan untuk memencilkan enzim protease sementara penulinan separa enzim tersebut telah dilakukan dengan penurasan gel melalui Sephadex G-200. Empat pecahan yang dimendakkan oleh 0 - 20%, 20 - 40%, 40 - 60% dan 60 - 80% penepuan amonium sulfat telah menunjukkan aktiviti proteolisis terhadap kasein. Kajian yang seterusnya telah dijalankan ke atas pecahan yang telah dimendakkan oleh 0 - 20% dan 20 - 40% penepuan amonium sulfat. Kajian ke atas prosedur pemencilan telah menunjukkan bahawa penambahan detergen natrium dodesil sulfat atau Triton

X-100 tidak dapat meningkatkan kecekapan proses pemencilan. Kajian suhu menunjukkan bahawa pecahan 0 - 20% dan 20 - 40% mempunyai suhu optimum 45 - 50 °C walaupun didapati tidak stabil pada suhu tersebut. Kedua-dua pecahan mempunyai lebih daripada satu pH optimum terhadap kedua-dua kasein dan albumin serum lembu. pH optimum itu terletak dalam julat pH asid kuat dan pH alkali kuat. Kajian perencatan menunjukkan bahawa kedua-dua pecahan 0 - 20% dan 20 - 40% mungkin mengandungi protease jenis sisteina walaupun kehadiran protease jenis aspartik tidak dapat diketepikan. Perjalanan pecahan 0 - 20% melalui Sephadex G-200 telah menghasilkan dua puncak protein yang mempunyai aktiviti proteolisis yang kemudiannya telah dilabelkan sebagai P1 dan P2 manakala pecahan 20 - 40% menghasilkan lima puncak aktiviti yang tidak dapat dipisahkan dengan baik. Kajian menggunakan elektroforesis gel poliakrilamida natrium dodesil sulfat menunjukkan yang pecahan 0 - 20% adalah lebih tulin secara perbandingan dan bahawa puncak P1 berkemungkinan besar adalah agregat protein. Dari pencirian enzim protease di dalam kedua-dua pecahan tersebut jelas menunjukkan bahawa enzim ini memang memainkan peranan dalam penghasilan perisa koko dan pelopornya semasa fermentasi koko.

CHAPTER I

GENERAL INTRODUCTION

Botanical Background of Cocoa

Cocoa, botanically known as *Theobroma cacao* Linneaus, is one of the 22 species that constitute the genus Theobroma. It is taxonomically classified under the family of Sterculiaceae under the order of Malvales. Out of the 22 species of Theobroma, cocoa is the only one of commercial value and it is divided into two main types, namely Criollo and Forastero. There is, however, a third type called Trinitario which is basically a cross between the first two types (Minifie, 1980; Cook, 1982; Wood and Lass, 1984).

Each cocoa tree cultivated from seeds produces three to five fan branches at the jorquette of the tree once the main trunk reaches a height of 4 to 5 feet. Flowering begins when the tree is 1½ years old and it occurs both at the main trunk and the fan branches. There are two peak flowering seasons per year and an adult tree can produce up to 10,000 flowers in one year of which only 10 to 40 of them will eventually mature to become pods (Cook, 1982; Wood and Lass, 1984).

The pod, which may attain a length of 6 to 10 inches and a diameter of 3 to 4 inches, contains about 20 to 40 seeds surrounded by a mucilaginous pulp when the pod is ripe. After the seeds are fermented and dried, they are commercially termed as cocoa beans, or cocoa when in bulk. Cocoa thrives well under a temperature of 18 to 32 °C and an annual rainfall of 1,250 to 2,800 mm, and most areas in Malaysia fulfil this climatic requirement (Minifie, 1980; Cook, 1982; Wood and Lass, 1984; Malaysian Cocoa Board, 1991).

Historical Background of Cocoa in Malaysia

Cocoa is not indigenous to Malaysia, but originated from the Upper Amazon basin in Latin America. It is possible that it reached Sabah as early as 1700. The earliest record of its occurrence in Peninsular Malaysia was by J.C. Koenig who reported seeing a fruiting tree in Malacca in 1778. In 1882, Von Donop reported having seen cocoa trees of at least 20 years old fruiting well in British North Borneo (now Sabah) (Cook, 1982; Wood and Lass, 1984; Malaysian Cocoa Board, 1991).

The first serious attempts at cocoa cultivation started with experimental plots at the Agricultural Research Station in Serdang, Selangor and at the Agricultural Experimental Station in Silam, Sabah. However, interest in the crop was minimal until after the Second World War when the government was

looking for other crops to supplement the contribution of rubber to the economy of the then Malaya, Sarawak and British North Borneo. Professor E.E. Cheesman was thus assigned to the task of assessing the prospects of cocoa growing in the three territories. As a result of his report, in 1950 Amelonado (Forastero) seedlings from West Africa were imported and tested on the volcanic soil of Tawau, Sabah and in observational plots at the Tarat Research Station, Sarawak (Wyrley-Birch, 1976; Malaysian Cocoa Board, 1991).

The first commercial cocoa estate was established in 1950 at Jerangau, Terengganu. This was followed by the Borneo Abaca Ltd. (now BAL Plantations), Sabah in 1955. The initial planting materials used were of the Amelonado type but in the mid-1950s, it was badly affected by a disease known as the vascular streak dieback caused by the fungus *Oncobasidium theobromae*. In 1957, the Quoin Hill Research Station was established by the Department of Agriculture Sabah and a programme on varietal improvement there produced the high yielding and disease tolerant Upper Amazon hybrid. This new hybrid subsequently replaced the Amelonado type (Wyrley-Birch, 1976; Wood and Lass, 1984; Malaysian Cocoa Board, 1991).

The development of cocoa was further boosted under the Coconut Replanting and Rehabilitation Scheme in Peninsular Malaysia whereby government subsidy was provided for the

purpose of growing cocoa as an intercrop of coconut. However, it was the high prices of cocoa beans in the late 1970s and early 1980s that contributed to the phenomenal expansion of cocoa cultivation throughout the country particularly in Sabah (Wood and Lass, 1984; Malaysian Cocoa Board, 1991). From Table 1, it can be seen that the total area of cocoa cultivation between 1975 and 1985 saw a 10-fold increase, from 30,280 hectares in 1975 to 303,897 hectares in 1985, and this increase was greatest seen in Sabah which was more than 15-fold from 9,823 hectares in 1975 to 172,713 hectares in 1985.

Cocoa Industry in Malaysia

At present, cocoa is Malaysia's third most important agricultural export crop after rubber and oil palm. In 1990, Malaysia produced 255,000 tonnes of cocoa beans and this placed Malaysia as the fourth largest producer of cocoa beans in the world after Cote d'Ivoire (Ivory Coast), Brazil and Ghana. Of that, a total of 162,618 tonnes, which represents about 65% of the total production, was exported, mainly to Singapore, the Federal Republic of Germany and the Netherlands, bringing in a total of M$448.5 million in foreign exchange. More recently, increasingly large quantities of beans are being processed locally and in 1990, 28,600 tonnes of cocoa butter valued at M$240 million and 10,102 tonnes of cocoa powder valued at M$24.5 million were exported (Malaysian Cocoa Board, 1991).

Table 1

Area under Cocoa in Malaysia (1960 - 1990)

Year	Area (ha)			
	Peninsular Malaysia	Sabah	Sarawak	Total
1960	577	1,170	-	1,747
1961	575	1,538	-	2,113
1962	585	1,942	-	2,527
1963	591	2,023	-	2,614
1964	664	2,145	-	2,809
1965	761	2,187	-	2,948
1966	822	2,643	-	3,465
1967	865	2,793	-	3,658
1968	1,124	3,117	-	4,241
1969	1,902	3,331	-	5,233
1970	3,362	4,019	-	7,381
1971	5,878	4,517	-	10,392
1972	8,984	5,447	880	15,311
1973	11,599	6,242	1,481	19,322
1974	13,634	8,126	2,313	24,073
1975	17,587	9,823	2,870	30,280
1976	20,796	11,673	3,342	35,811
1977	29,635	14,994	3,850	48,479
1978	34,268	22,097	4,557	60,922
1979	45,168	37,438	6,385	88,991
1980	57,345	57,984	8,526	123,855
1981	64,618	83,455	10,711	158,784
1982	82,185	114,474	12,740	209,399
1983	83,949	132,729	14,402	231,080
1984	89,163	159,288	17,059	265,510
1985	106,932	172,713	24,252	303,897
1986	105,908	184,477	31,949	322,334
1987	122,772	196,944	43,293	363,009
1988	141,750	204,466	53,675	399,891
1989	147,904	208,500	54,700	411,104
1990	148,400	211,300	60,600	420,300

(Source: Malaysian Cocoa Board, 1991)

However, the Malaysian cocoa industry is not without its problems. Malaysian cocoa beans is being sold at a discount of £75 per tonne at the London Terminal Market and, when a shortage of African beans occurs, the price difference between Malaysian and Ghanaian beans can go up to more than £200 per tonne. This disparity in prices is due to some weaknesses in the quality of Malaysian beans, namely, large variations in bean sizes, high shell content, weak chocolate flavour, smoky off-flavour and high acidity of the beans (Lee, 1989).

Consequently, many studies have been or are being carried out to study the weaknesses and subsequently, to minimise them. Both governmental statutory bodies and companies in the private sector are involved in cocoa research and development (Lewis and Lee, 1985). Varietal improvement and clonal selection studies have been conducted by the Department of Agriculture Peninsular Malaysia (until 1969), the Department of Agriculture Sabah, the Malaysian Agricultural Research and Development Institute (MARDI) (since 1969), Sime Darby Plantations, and BAL Plantations. In addition to that, research on the fermentation and processing of cocoa beans have been undertaken by the Department of Agriculture Sabah, MARDI, and also by Sime Darby Plantations. Since its establishment on July 19, 1989, the Malaysian Cocoa Board has been responsible for coordinating all activities relating to the cocoa industry in Malaysia including

the area of research and development (Malaysian Cocoa Board, 1991).

Relevance and Importance of Present Study

In spite of the extensive and concerted efforts that had been invested into cocoa research and development, studies on the role of enzymes in the production of cocoa flavour and flavour precursors have been largely ignored and this is more apparent in the local scene. Therefore, there is an urgent need to look into this neglected research approach and to supplement the work that has been carried out in other areas.

The enzyme chosen for this study in this project was protease because the enzymatic proteolysis of cocoa bean proteins into amino acids and peptides is believed to be one of the major pathways for the production and formation of cocoa flavour precursors. The study was initiated with the intention of isolating and characterising protease from cocoa beans. In the course of the investigation, four fractions of proteases were isolated. For the purpose of this project, the scope was limited to two of the fractions whereby further studies were conducted on these two fractions and their characteristics were compared with each other.