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DETECTION OF CELLULAR PRION PROTEIN IN ANIMALS OF ECONOMIC IMPORTANCE OF THAILAND

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พริออนโปรตีนเป็นตัวก่อโรคเกี่ยวกับระบบประสาทซึ่งเป็นโรคในกลุ่ม transmissible spongiform encephalopathies (TSEs) ในคนและสัตว์เลี้ยงลูกด้วยนมหลายชนิดซึ่งมีอันตรายถึงแก่ชีวิต งานวิจัยนี้ได้ทำการผลิตพอลีโคลนัลแอนติซีราต่อสายเปปไทด์สังเคราะห์ 3 สายซึ่งสังเคราะห์ขึ้นตามลำดับกรดอะมิโนใน 3 บริเวณที่แตกต่างกันบนสายพริออนโปรตีนของแกะโดยเปปไทด์ทั้ง 3 สายมีตำแหน่งของกรดอะมิโนดังนี้ 45-56 (PI) 145-163 (PII) และ 209-228 (PIII) ซึ่งสังเคราะห์ขึ้นโดยใช้ระบบ multiple antigenic peptides (MAPs) เพื่อใช้ในการตรวจสอบหาเซลล์ลูลาร์พริออนโปรตีนในสัตว์เศรษฐกิจของประเทศไทยคือปลาดุก หมู และวัว โดยแบ่งหนูพันธุ์ BALB/cA เป็น 3 กลุ่มๆละ 3 ตัวแล้วฉีดสายเปปไทด์สังเคราะห์แต่ละสายให้กับหนูแต่ละกลุ่ม พบว่าเปปไทด์สังเคราะห์ทั้ง 3 สายสามารถกระตุ้นให้สร้างแอนติบอดีได้ ตรวจวัดความสามารถในการจับของพอลีโคลนัลแอนติซีราที่ได้จากหนูแต่ละตัวกับเปปไทด์สังเคราะห์แต่ละสายโดยวิธี Indirect Enzyme-linked immunosorbent assay (ELISA) พบว่ามีค่า antibody titers (OD_{50}) ของพอลีโคลนัลแอนตีซีราต่อเปปไทด์สังเคราะห์ I (Ab PI) II (Ab PII) และ III (Ab PIII) เท่ากับ 1:1,600 1:512,000 และ 1:1,683,400 ตามลำดับ พริออนโปรตีนถูกสกัดจากสมองของปลาดุก หมู และวัวโดยการละลาย membrane ด้วยสาร Zwittergent 3-12 (ZW 3-12) แล้วถูกแยกด้วยการปั่นเหวี่ยงที่ความเร็วรอบต่างกัน (differential centrifugation) และทำบริสุทธิ์บางส่วนด้วยวิธี immobilized metal ion affinity chromatography (IMAC) โดยอาศัยความสามารถในการจับคอปเปอร์อิออน (Cu^{2+} ion) ของพริออนโปรตีน ตรวจสอบโปรตีนที่สกัดได้โดยการย้อมด้วย Coomassie blue หรือด้วยเทคนิค immunoblot หลังจากย้อมพริออนโปรตีนด้วยพอลิโคลนัลแอนติซีราที่ผลิตได้ทั้ง 3 ตัวหรือมอนอโคลนัลแอนติบอดีทางการค้า (mAb L42) พบว่าในสารสกัดหยาบของสมองหมู และวัวมีแถบโปรตีนที่มีน้ำหนักโมเลกุลประมาณ 30 และ 40 กิโลดาลตัน ในขณะที่ในสมองปลาพบแถบโปรตีนที่มีขนาดประมาณ 40 และ 42 กิโลดาลตัน เมื่อผ่านขั้นตอนการทำบริสุทธิ์บางส่วนด้วยวิธี IMAC แล้วย้อมด้วยพอลิโคลนัลแอนติซีรา Ab PII พบว่าสามารถแยกพริออนโปรตีนได้สองส่วน คือในส่วนของการชะด้วย 10 mM imidazole ซึ่งพบแถบโปรตีนที่มีขนาดเท่ากับแถบโปรตีนที่ได้จากสารสกัดหยาบในตัวอย่างสัตว์ทั้งสามชนิด แต่ในส่วนของการชะด้วย EDTA พบแถบโปรตีนที่มีน้ำหนักโมเลกุลประมาณ 21 กิโลดาลตัน ในตัวอย่างของหมูและวัวแต่ไม่พบในปลา

ภาควิชา ……ชีวเคมี……	ลายมือชื่อนิสิต …….
สาขาวิชา……ชีวเคมี……	ลายมือชื่ออาจารย์ที่ปรึกษา ……
ปีการศึกษา……..2546……..	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม …….

4372423023 : MAJOR BIOCHEMISTRY
KEY WORD : cellular prion protein (PrP^C)/ polyclonal antisera/ Multiple Antigenic Peptides (MAPs)/ immunoblot assay
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Prion protein is the infectious causative agent of a fatal neurodegenerative disease belonging to a group of transmissible spongiform encephalopathies (TSEs) in various mammalian species including human. In this research polyclonal antisera were raised against three synthetic peptides corresponding to amino acids sequences in three different regions of sheep PrP; 40-56 (PI), 145-163 (PII) and 209-228(PIII). These peptides were custom-synthesized using an automated peptide synthesizer and multiple antigenic peptides (MAPs). The polclonal antisera were used to detect the cellular prion protein in animals of economic importance of Thailand: catfish, pigs, and cows. In this study nine BALB/cA mice were divided into 3 groups, each group was separately immunized with synthetic peptides PI, PII, and PIII. The antiserum elicited by each peptide displayed antibody binding to the homologous peptide. Antibody titers (OD_{50} values) were determined by testing in an enzyme-linked immunosorbent assay (ELISA). Antisera against sequence PI (Ab PI), PII (Ab PII), and PIII (Ab PIII) showed the antibody titer at dilutions of 1:1,600, 1: 51,200 and 1:1,638,400, respectively. PrP^C was purified from microsomal fraction by detergent extraction with Zwittergent 3-12 (ZW 3-12), separated by differential centrifugation and partially purified by immobilized Cu^{2+} ion affinity chromatography (IMAC) owing to the binding capacity of PrP to Cu^{2+} ions. Determination by Coomassie blue staining and immunostaining with mouse polyclonal antisera and monoclonal antibody (mAb L42) showed protein bands at 30 and 40 kDa in crude brain homogenates of the pig and the cow, while crude brain homogenate of fish yielded protein bands at 40 and 42 kDa. During the partial purification by IMAC, PrP^C was eluted by either imidazole or EDTA. When subjected to immunostaining by Ab PII, this imidazole eluent showed the same protein bands as found in crude brain homogenates of fish, the pig, and the cow. The EDTA eluent, however, showed an immunostained protein band at 21 kDa in cases of the pig and the cow but not in the case of fish.

Department …….Biochemistry…….. Student's signature……..

Field of study…..Biochemistry……. Advisor's signature…….….

Academic year……..2003…… Co-advisor's signature ….……..……..

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ABBREVIATIONS

BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
^{O}C	degree Celsius
CFA	complete Freund's adjuvant
cm	centrimetre
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linkedimmunosorbent assay
Fmoc	9-fluorenylmethyl-oxycarbonyl
FPLC	fast protein liquid chromatography
IFA	incomplete Freund's adjuvant
IgG	Immunoglobulin G
IMAC	immobilized metal-ion affinity chromatography
kDa	kiloDalton
l	liter
µg	microgram
µl	microlitre
µM	micromolar
M	mole per liter (molar)
mA	milliampare
mAb	monoclonal antibody
MAP	multiple antigenic peptide
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MW	molecular weight
ng	nanogram
nm	nanometer
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosephate buffer saline

PMSF	phenyl methyl sulfonyl fluoride
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{Sc}	scrapie prion protein
SDS	sodium dodecyl sulfate
TPCK	*N*-tosyl-L-phenylalanine chloromethyl ketone
TSE	transmissible spongiform encepalopathy
UV	ultraviolet
V	voltage
v/v	volume by volume
w/w	weight by weight
ZW 3-12	*N*-Dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate

CHAPTER I

INTRODUCTION

1.1 Prions

Prions are transmissible infectious agents that differ from bacteria, fungi, parasite, viroids, and viruses respect to their structures and the diseases that they cause (Prusiner, 1991). The prion protein causes the group of invariably fatal neurodegenerative disorders called transmissible spongiform encephalopathies (TSEs) (Prusiner, 1998; Hope *et al.*, 2000) by and entirely novel mechanism. Prion diseases (***Table 1.1***) may present as most (80%) sporadic disorders, some (19%) genetic or inherit, and a small proportion (1%) of transmission of the infectious agent between mammals (Rudd *et al.*, 2001). These diseases that cause in mammalian species include bovine spongiform encephalopathy (BSE or mad cow disease) in cattle, scrapie in sheep and goat, Creultfeldt- Jakob disease (CJD) and Kuru in human, and chronic wasting disease (CWD) in deer and elk (Caughey, 2001). The nature of the infectious agent of prion diseases has not been fully elucidated (Bolton and Bendheim, 1991). However, they seem to be the result of a conformational change on the cell surface and/or in an endocytic pathway (Maiti and Surewicz, 2001), involving a transition from α-helix to β-sheet structure (Pan *et al.*, 1993), in the normal cellular isoform of prion protein (PrP^{C}) to the scrapie isoform (PrP^{Sc}) which is the main factor responsible for accumulation in the brain of affected animals (Bolton *et al.*, 1982). Animals affected with these diseases show neurodegenerative disorders after a long incubation period and die (Yokoyama *et al.*, 1995).

Table 1.1 The prion diseases (Prusiner, 1998)

Diseases	Host	Mechanism of pathogenesis
Kuru	Fore people	Infection through ritualistic cannibalism
iCJD	Humans	Infection from people-contaminated HGH, dura mater grafts, etc.
vCJD	Humans	Infection from bovine prions?
fCJD	Humans	Germ-line mutations in PrP-gene
GSS	Humans	Germ-line mutations in PrP-gene
FFI	Humans	Germ-line mutations in PrP-gene (D178N, M129)
sCJD	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}
FSI	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}
Scrapie	Sheep	Infection in genetically susceptible sheep
BSE	Cattle	Infection with prion-contaminated MBM
TME	Mink	Infection with prions from sheep or cattle
CWD	Mule, deer, elk	Unknown
FSE	Cats	Infection with prion-contaminated bovine tissues or MBM

iCJD, iatrogenic CJD; vCJD, varian CJD; fCJD, familial CJD; sCJD, sporadic CJD; GSS, Gerstmann-Straussler-Sheinker disease; FFI, fatal familial insomnia; FFI, fatal sporadic insomnia; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; FSE, feline spongiform encephalopathy; HGH, humans growth hormone; MBM, meat and bone meal.

Pathogenesis studies on these diseases and the chemical form of the transmissible agent are the focus of interesting history. In 1939, Harris first demonstrated that the disease was experimentally transmitted to goats by intraocular injection. Gajdusek and his colleagues (1957) described a strange neurodegenerative fatal disease was transmitted by ritual cannibalism among the group of Fore people of Papua New Guinea called Kuru. After that, William Hadlow (1959) indicated the neuropathological similarities between Kuru and scrapie. Subsequent work demonstrated that Kuru and CJD were transmissible to non-human primate, chimpanzees by intracerebrally inoculation (Gajdusek *et al.,* 1966). There is remarkable species specificity to the transmission of prion diseases; first showed by Pattison (1966) that manifest it in prolong incubation period upon first passage between species. This effect is observable even between closely related species such as mice and hamster (Scott *et al.,* 1989).

The unusual properties of the infectious agent become the focus of attention beginning in the 1960s. According to the “prion only” hypothesis that proposed by Stanley Prusiner in the early 1980s, the infectious pathogen that causes human and animal spongiform encephalopathies was composed exclusively of a single kind of protein molecule designated PrP^{Sc} with devoid of nucleic acid (Prusiner, 1982). Most procedures that modify nucleic acid did not alter scrapie infectivity (Harris, 1999). Several investigators found that scrapie agent was extremely resistant to inactivation by both ultraviolet and ionizing radiation, in addition, reagent specifically modifying or damaging nucleic acid, such as nucleases, psoralens, hydroxylamine, and Zn^{2+} ion, do not alter scrapie infectivity in homogenates, microsomal fraction (Prusiner, 1982), purified prion rod preparations, or detergent-lipid-protein complexes (Prusiner, 1991).

Prion diseases have now become an interest subject because of the potential risks they pose to public health (Harris, 1999). Since 1986, more than 28,500 cattle have died of BSE (mad cow disease) in Great Britain, and a much larger number of animals have probably been infected (Prusiner, 1997). In Japan, there is a sporadic occurrence of scrapie in sheep, but no outbreak of BSE has been observed (Yokoyama *et al.*, 1995). Many investigators believed that BSE resulted from the feeding scrapie-contaminated meat and bone meal (MBM) that derived from scrapie agent of sheep and goat to cattle. Although a government-imposed feed ban in 1988 has significantly reduced the critical of the disease, it has had a major economic and political impact throughout Europe.

1.2 Expression of PrP

PrP^C, a host-encoded normal cellular protein, is a sialoglycoprotein that is attached to the outer of the plasma membrane via a C-terminal glycosyl-phosphatidylinositol (GPI) anchored (Stahl *et al.*, 1990) with a molecular mass of 33-35 kDa as shown in ***Figure 1.1***. It is abundant expressed in the neurons and glia of the brain and spinal cords, as well as in several peripheral tissues and in leukocyte (Caughey *et al.*, 1988; Bendheim *et al.*, 1992; Moser *et al.*, 1995). Further, in the brain, PrP^C is particularly localized to synaptic membranes, most likely the pre-synaptic domain (Vassallo and Hern, 2003). A single chromosomal encodes it gene (*prnp*) in all mammals and the localization of this PrP gene is on the short arm of human chromosome 20 and the same region of mouse chromosome 2 (Prusiner, 1991). The entire open reading frame (ORF) of all known mammalian and avian PrP gene is located within a single exon which codes for a protein approximately 250

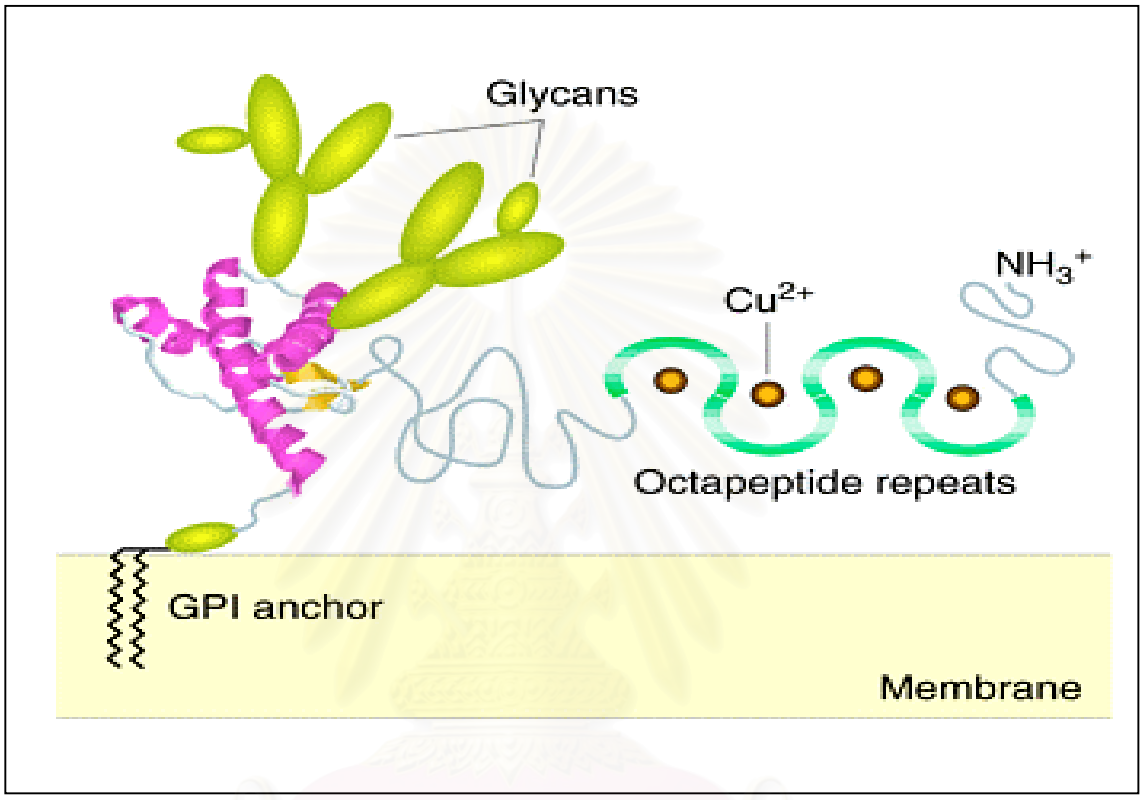

Figure 1.1 Model of PrP^C domains (Caughey, 2001). The folded C-terminal portion of PrP^C that contains the short β-sheet strands (yellow arrows) and the α-helices (pink) is based on a model derived from the nuclear magnetic resonance (NMR)-based coordinates of residues124-228 of hamster PrP (Donne *et al.*, 1997). The remainder of the molecule appears, by, NMR, to be flexible disordered. GPI, glycosylphosphatidylinositol moiety; PrP^C, cellular prion protein.

amino acids (Martin *et al.*, 2001). As recently reviewed by Groschup (1997), the PrP genes of mouse, rat, and Syrian hamster code for 254 amino acids (Oesch *et al.*, 1985; Locht *et al.*, 1986); those of sheep, goat and cattle code for 256 amino acids (Goldmann *et al.*, 1991; Martin *et al.*, 1994); those of pig and mink code for 257 amino acids (Kretzschmar *et al.*, 1992; Martin *et al.*, 1995); that of human code for 253 amino acids as shown in ***Figure 1.2***. The putative chicken PrP, which displays considerable sequence differences to PrP of mammals, comprises 273 amino acids (Gabrial *et al.*, 1992). Several domains of the protein are highly conserved and show high homology among many species (Basler *et al.*, 1986; Robakis *et al.*, 1986), the similarity is about 85 to 97% among mammals and the comparison between primates and humans showed amino acid identity ranging from 92.9 to 99.6% (Martins *et al.*, 2001). Only about 5% amino acid differences exist among the known sequences of PrP from mouse, hamster, sheep, and cattle (Yokoyama *et al.*, 1995), suggesting an important physiological role for this protein In human amino acid mutations which related to mutation of PrP gene (*PRPN*) are frequently associated with prion diseases (***Table 1.2***). More than 20 mutations which linked to specific mutations in the PrP gene are now known to genetic cause the inherited human prion disease (Prusiner, 1998), such as the P102L mutation of GSS, E200K of CJD, and M129/D178N of FFI (Swientnicki *et al.*, 2001). PrP mRNA is first detectable in the brain of mice and chickens beginning in embryogenesis, and its level increases as development proceeds but the levels are highly regulated and can be stimulated by nerve growth factor (Kretzschmar *et al.*, 1986; Mobley *et al.*, 1988). Although PrP mRNA is constitutively expressed in the brains of adult animals, it is regulated during development (Oesch *et al.*, 1985). In the adult central nervous system, PrP and its mRNA are widely distributed, with particular concentrations in necocortical and

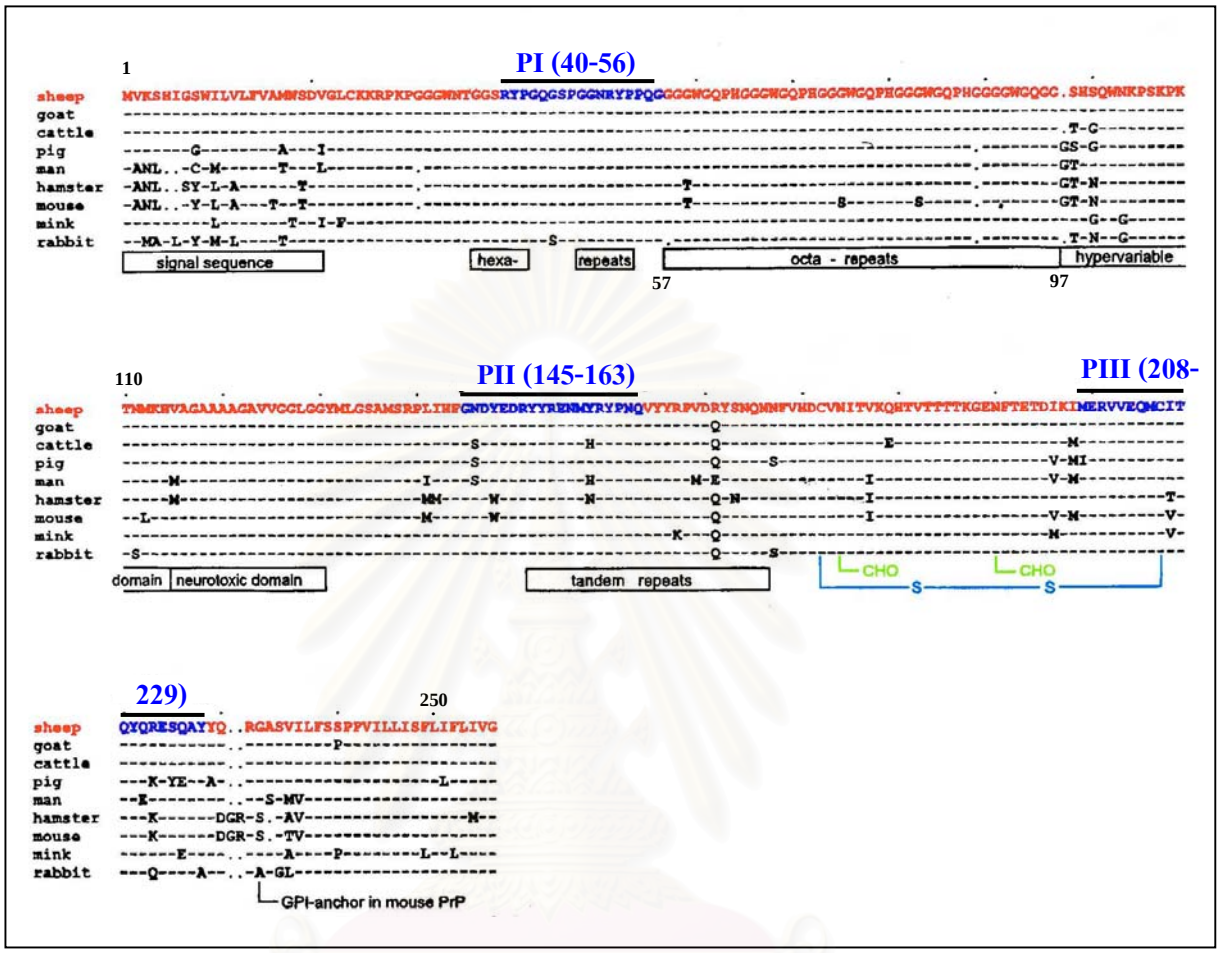

Figure 1.2 Alignment of PrP amino acids sequences of different species (Groshup et al., 1997). Alignment of the known amino acid sequences of sheep, goat, cow, pig, man, Syrian hamster, mouse, mink, and rabbit. Amino acid positions are numbered according to ovine PrP. The sites of N-glycosylation are amino acid sequence 184 and 200, and the cysteine disulfide loop is amino acid sequence 182 and 217. PI, PII, and PIII are the three peptide sequences that were synthesized and used for raising mouse antibodies as described in Chapter II.

Table 1.2 Mutations of the chromosome 20 'prion' gene associated with inherited forms of transmissible spongiform encephalopathy (http://www.cjdinsight.org)

Mutation	Disease Phenotype
Octa-repeat insertion of 24, 48, 96, 120, 144, 168, 192, or 216 base pairs between codons 51 and 91	CJD, GSS, or atypical dementias
P102L (Pro Leu)	GSS: classical ataxic form
P105L (Pro Leu)	GSS: spastic paraparetic variant
A117V (Ala Val)	GSS: pseudobulbar variant
G131V (Gly Val)	GSS: classical ataxic form
Y145* (Tyr Stop)	Alzheimer-like dementia
D178N (Asp Asn)	CJD (129V on mutant allele)
D178N (Asp Asn)	FFI (129M on mutant allele)
V180I (Val Ile)	CJD
T183A (Thr Ala)	Alzheimer-like dementia
H187R (His Arg)	GSS: classical ataxic form
F198S (Phe Ser)	GSS with neurofibrillary tangles
E200K (Glu Lys)	CJD
D202N (Asp Asn)	GSS with neurofibrillary tangles
V203I (Val Ile)	CJD
R208H (Arg His)	CJD
V210I (Val Ile)	CJD
E211Q (Glu Gln)	CJD
Q212P (Gln Pro)	GSS with Lewy bodies
E217R (Glu Arg)	GSS with neurofibrillary tangles
M232R (Met Arg)	CJD

CJD = Creutzfeldt-Jakob disease;

GSS = Gerstmann-Straussler-Scheinker syndrome;

FFI = fatal familial insomnia.

hippocampal neurons, cerebella Purkinje cell, and spinal motor neurons (DeArmond *et al.*, 1987). In the septum, PrP mRNA and choline acetyltransferase were found to increase in parallel during development (Mobley *et al.*, 1988).

1.3 Function of PrP^C in vivo

Defining the physiological role of PrP^C may be relevant to understanding of how prion cause the neurodegenerative diseases, since the protein may fail to perform its normal function when it is converted to the pathological isoform, designated PrP^{Sc} (Harris, 1999). The normal function of PrP^C remains unknown exactly, although its localization on the cell surface makes it a potential candidate for a ligand uptake, cell adhesion and recognition molecule or a transmembrane signaling molecule (Martins *et al.*, 2001), and the possible role in synaptic function, circadian rhythm and promoting genetic diversity have been suggested (Rudd *et al.*, 2001). In addition to, PrP^C is expressed constitutively in skeletal muscle and is suggested to play a role in regulating skeletal muscle resistance to oxidative stress (Mitchell, 1999). In several evidences PrP^C was postulates to be a major copper-binding protein in brain based on the observation that the normal copper content in membrane fractions derived from the brains of mice which carry disrupted PrP gene ($PrP^{-/-}$) drops to 5-50% (Quaglio *et al.*, 2001). The majority of research on prion function has focused on cerebellar cells as model system. Brown and co-worker (1997) using cerebellar cell cultures from mice expressing different levels of PrP^C, demonstrates that cells with high levels of PrP^C have an increasing resistance to oxidative stress compared to $PrP^{-/-}$ cells (Martin *et al*, 2001). Recently, there is increasing evidence supporting a potential role for PrP^C in the metabolism of copper and moreover that this metal stimulates PrP^C endocytosis. The copper ions bind with low micromolar affinity to the octapeptide

repeats region in the N-terminal half of mammalian PrP^C which in mouse contains four copies of the sequence PHGG(G/S)WGQ and one copy of the sequence PQGGTWGQ (Viles *et al.,* 1999; Miura *et al.,* 1999). Spectroscopic data have suggested a bridged arrangement of coordinating histidine imidazole nitrogens binding four Cu^{2+} ions per PrP^C molecule (Martins *et al.,* 2001) as shown in ***Figure 1.3***. In addition, copper rapidly and reversibly stimulates endocytosis of PrP^C from the cell surface, raising the possibility that PrP^C normally serves as a part of a system to transport copper in and out of cells. The octarepeats share a common sequence (PHG) with the histidine-rich glycoprotein, a protein involved in plasma copper transport (Harris, 1999). The brain of PrP null-mice displayed a reduced content of membrane-associated copper and a decrease in activity of copper-zinc superoxide dismutase (Cu,Zn SOD) (Hornshaw *et al.*, 1995; Brown *et al.*, 1997). In fact, copper chelate affinity chromatography has been used in the purification of PrP^C (Stockel *et al.*, 1998).

Disturbances in copper homeostasis leading to CNS dysfunction are well documented in humans and animals. Some neurodegenerative diseases such as Menkes' syndrome, Wilson's disease, amyotrophic lateral sclerosis and Alzheimer's disease are resulted in the altered copper transport and homeostasis (Waggoner *et al.*, 1999). The evidence that PrP^C has a role in copper metabolism may be important in understanding the pathogenesis of prion diseases, since loss of this copper- related function (as a result of conversion to PrP^{Sc}) could help explain some features of these disorder (Martins *et al.,* 2001). Interestingly, early studies have revealed that cuprizone, a copper-chelating agent, induces neuropathological changes in mice, if they are treated with cuprizone, they develop spongiform degeneration and reactive

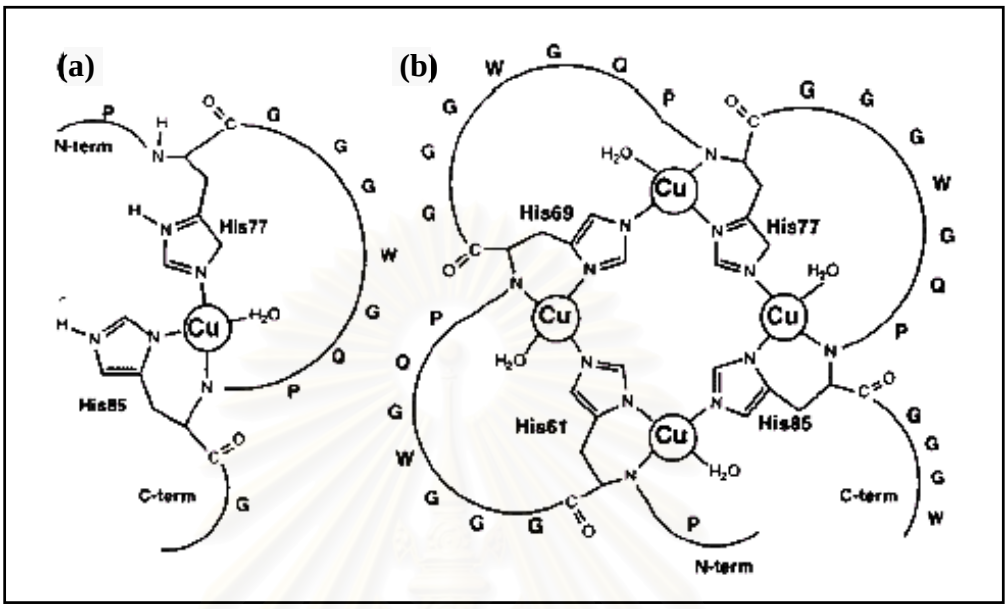

Figure 1.3 Plausible structures for the complex of Cu (II) with PrP (Vile et al., 1999). (a) Structure complex of Cu (II) with PrP (76-86), and (b) Structure for the bridge complex of four Cu (II) ions with PrP (58-91).

astrocytic gliosis very similar to those found in prion diseases, suggesting a role for copper in these diseases (Pattison *et al.*, 1971).

1.4 Biosynthesis of PrP

Like other membrane proteins, PrP^C is synthesized in rough endoplamic recticurum (ER) and transits to Golgi on secretory pathway to the cell surface where it preferentially accumulates in cholesterol-rich membrane domains called rafts (Morillas *et al.*, 2001). During its biosynthesis, PrP^C is subject to several kinds of posttranslational modification as shown in ***Figure 1.4***, including (1) cleavage of the NH_2-terminal signal peptide of 22 amino acids, (2) addition of N–linked oligo-saccharide chains at two sites, Asn-181 and Asn-197, (3) formation of a disulfide bond between Cys-179 and Cys-214, and (4) cleavage of 23-Cterminal residues upon the addition of glycosylphosphatidylinositol (GPI) anchor to Ser-231 (Donne *et al.*, 1997). In the mature form of human prion protein, after removal of NH_2- and C-terminal signal sequence, the mammalian cellular PrP contains 210 amino acid residues, from 23 to 230 (Kozin *et al.*, 2001). The N-linked oligosaccharide chains added initially in the ER are of the high-mannose type and are sensitive to digestion by endoglycosidase H: these are subsequencely modified in the Golgi yield complex-type chains that contain sialic acid and are resistant to endoglycosidase H (Caughey *et al.*, 1989). N-linked glycosylation is often essential for the folding, stability, intracellular transport, secretion, and function of glycoprotein (Walmsley *et al.*, 2001). The GPI anchor, which is added in the ER after cleavage of the 23 amino acids from C-terminal hydrophobic segment, has a core structure common to other glycolipid-anchored proteins, consisting of an ethanolamine residue amide-bonded to

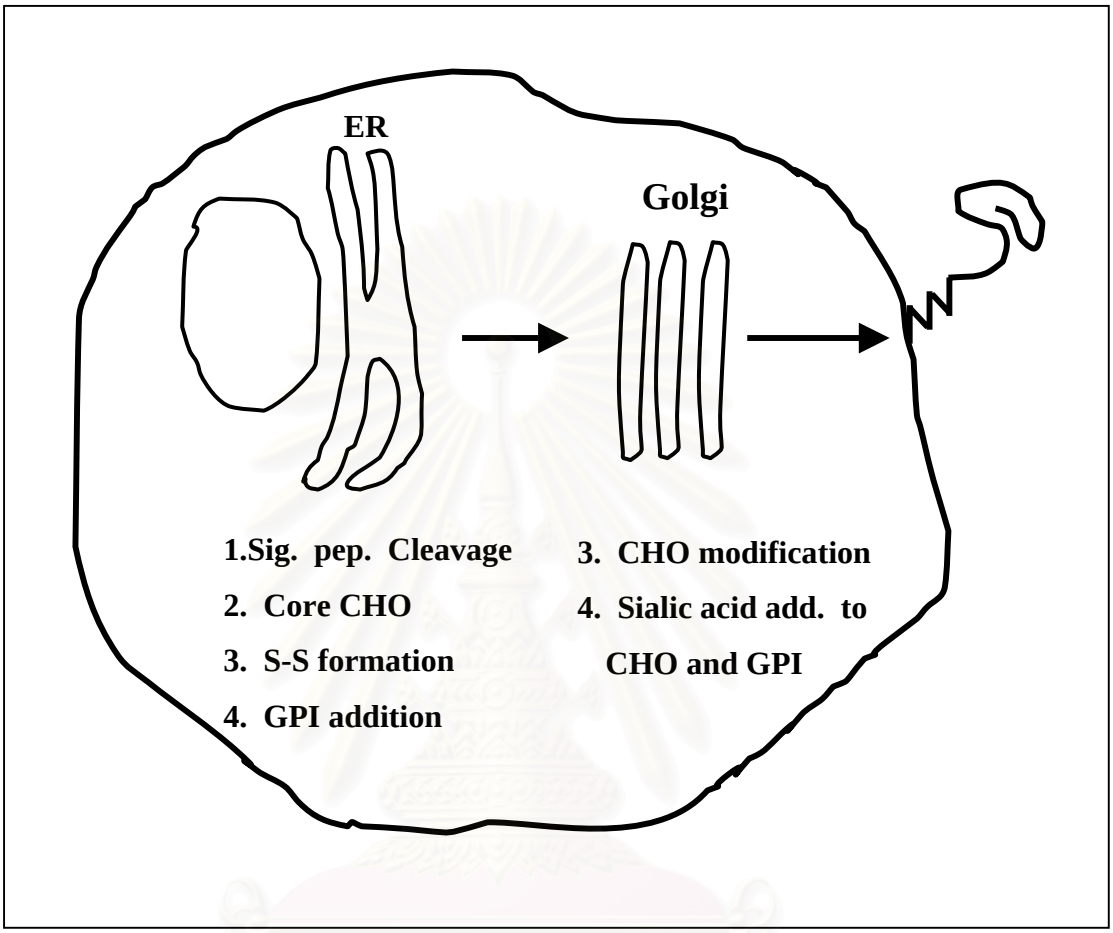

Figure 1.4 Steps in biosynthesis of PrP^C, CHO, oligosaccharide; S-S, disulfide bond; Sig.pep., signal peptide (Harris, 1999).

the C-terminal amino acid, three mannose residues, and unacetylated glucosamine residue, and a phosphatidylinositol (PI) molecule which is embedded in the outer of the lipid bilayer. There is evidence to suggest that the GPI anchor is important in the conversion of PrP^{C} to PrP^{Sc}. In vitro, treatment of cells with phospholipase C (PLC), an enzyme that hydrolyzes the GPI anchor, inhibit the production of PrP^{Sc} (Caughey and Raymond, 1991).

1.5 Structure and properties of PrP

The mammalian PrP gene encoded a protein of approximately 250 amino acids that contains several distinct domains, including an N-terminal signal peptide, a series of five proline and glycine-rich octapeptide repeats, a central hydrophobic segment that is highly conserved, and a C-terminal hydrophobic region that is a signal for addition of a glycosylphosphatidylinositol (GPI) anchor (Harris, 1999) as shown in ***Figure 1.5***. The deduced amino acid composition is given in ***Table 1.3***. Both PrP isforms, PrP^{C} and PrP^{Sc} contain a disulfide (S-S) bond between Cys179 and Cys214 and asparagine-linked glycosylation (CHO) occur at residues 181 and 197 according to human prion protein (Prusiner, 1991). While the N-terminal segment 23-120 of PrP^{C} is flexibly disordered “tail” and its C-terminal residues 121-231 form a globular domain with three α-helices comprising the residues 144-154, 173-194, and 200-228 and a short antiparallel β-stranded-sheet comprising the residues 128-131, and 161-164 according to human PrP (hPrP) (Zahn *et al.*, 2000) (***Figure 1.6***). The first turn of the second helix and the last turn of the third helix are linked by the single disulfide bond in the protein (Riek *et al.*, 1996). Within the globular domain, three polypeptide segments showed increased structural disorder; i.e., a loop of residues 167-171, the

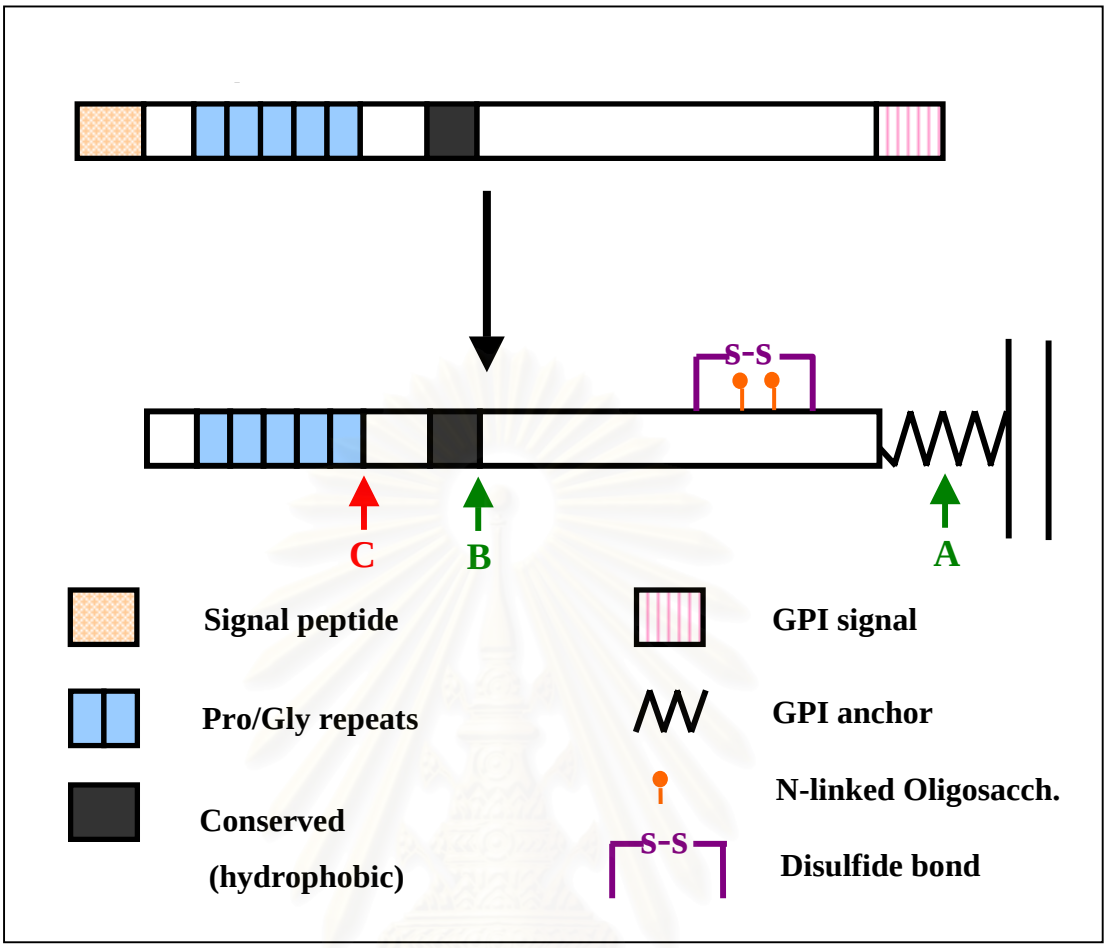

Figure 1.5 Structure and posttranslational processing of PrP (Harris, 1999). Structure of the primary translation product mammalian PrP (top). Structure of the mature protein (bottom). The GPI anchor attaches the polypeptide chain to the membrane. Arrow A and B indicate the positions of cleavage sites in PrP^{C}, and arrow C indicates a cleavage site in PrP^{Sc}. Site A lies within the GPI anchor, between the diacylglycerol moiety and the ethanolamine residue that is attached to the C-terminal amino acid. Site B lies near position 110, and site C lies near position 89.

Table 1.3 Amino acid composition of the complete PrP protein of Golden Syrain Hamster predicted from the genomic nucleotide sequence (Basler et al., 1986)

Amino acid	Number	Percent
Ala	12	5
Arg	11	4
Asn	16	6
Asp	7	3
Cys	3	1
Glu	7	3
Gln	16	6
Gly	43	17
His	9	4
Ile	7	3
Leu	12	5
Lys	11	4
Met	12	5
Phe	7	3
Pro	17	7
Ser	12	5
Thr	15	6
Trp	10	4
Tyr	3	5
Val	14	6

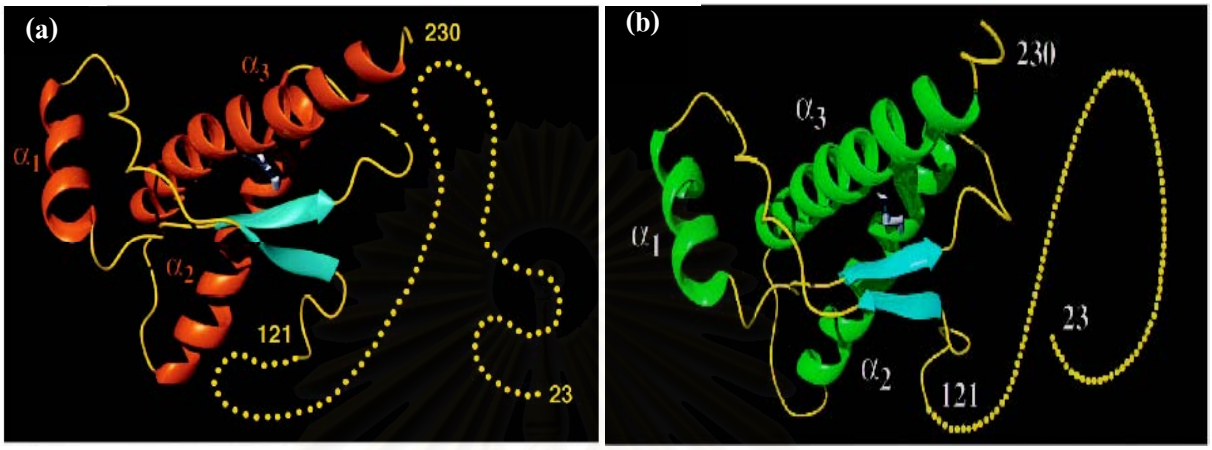

Figure 1.6 Cartoon of the three-dimensional structure of the intact (a); human prion protein, hPrP(23-231) (Zahn et al., 2000) and (b); bovine prion protein, bPrP(23-230) (Garcia et al., 2000). The helices are orange and green, respectively, the β-strands are cyan, the segments with nonregular secondary structure within the C-terminal domain are yellow, and the flexibly disordered "tail" of residues 23-231 is represented by yellow dot.

residues 187-194 at the end of helix 2, and the residues 219-228 in the C-terminal part of helix 3 (Zahn *et al.*, 2000). The species variations in the primary, secondary, and tertiary structure of the cellular isoform of PrP has previously been implicated in intermolecular interactions related both to the species barrier for infectious transmission of prion disease and to immune reaction (Zahn *et al.*, 2000). Fourier-transform infrared (FTIR) spectroscopy demonstrated that PrP^{C} has high α-helix content (42%) and little or no β-sheet (3%), and these findings were confirmed by circular dichroism measurement. In contrast, the β-sheet content of PrP^{Sc} was 43% and the α-helix 30 % as measured by FTIR (Pan *et al.*, 1993). According to the prion theory, PrP^{C} converts into PrP^{Sc} autocatalytically (Prusiner, 1982), which is thought to occur intracellularly after PrP^{C} or a precursor exits from the Golgi and transits to cell surface but before PrP^{Sc} is deposited in lysosome (Pan *et.al*, 1992). The transition between PrP^{C} and PrP^{Sc} is clearly a posttranslational process (Basler *et al.*, 1986; Borchelt *et al.*, 1990) without any detectable covalent modifications to the protein molecule, whereas the molecular mechanism for this change remain unknown (Morillas *et al.*2001). Both PrP isoforms seem to possess the same covalent structure and these two proteins have the same amino acid sequence (Zan *et al.*, 2000) but differ substantially in conformation; its secondary and tertiary structure (Liu *et al.*, 1999). Although the two isoforms of PrP have identical chemical properties but they have dramatically different biophysical properties: (*i*) PrP^{Sc} is insoluble in detergents, while PrP^{C} is readily solubilized under non denaturing condition (Meyer *et al.*, 1986); (*ii*) PrP^{Sc} is partially hydrolyzed by proteases to form a fragment designated PrP 27-30, while PrP^{C} is completely degraded under the same conditions (Oesch *et al.*, 1985); (*iii*) PrP^{Sc} accumulates, whereas PrP^{C} is monomeric and turns over rapidly; and (*iv*) the pattern of PrP^{Sc} accumulation in brain are distinct from the distribution of PrP^{C}

(Taraboulos *et al.*, 1992). Some properties of cellular and scrapie PrP in hamster was summarized in ***Table 1.4***. The protease resistance core of PrP^{Sc} designated PrP27-30 that removed the 67 NH_2-terminal amino acids, polymerizes into rod-shaped structures which are indistinguishable from many purified amyloids both ultrastructurally and tinctorially (Prusiner *et al.*, 1983). In the brains of some, but not all, animals and humans that have died of prion diseases, amyloid plaques are found which contain PrP, as determined by immunostaining and Edman protein sequencing studies (Pan *et al.*, 1993). PrP 27-30 polymerizes into amyloid suggests that it might have a β-pleated sheet structure (Prusiner *et.al*, 1983), since all amyloids studied, to date, have been found to have this structure (Pan *et.al*, 1993). About 50% of the secondary structure of PrP 27-30 is β-sheet, as measured by Fourier-transform infrared (FTIR) spectroscopy (Caughey *et.al*, 1991, and Gasset *et.al*, 1993). Two-third of the β-sheet content of PrP 27-30 is low-frequency (LF) β-sheet, which often reflects intermolecular associations that are a characteristic of amyloids (Gasset *et.al*, 1993). Determination of the conformations of PrP^{C} and PrP^{Sc} is important understanding the pathogenesis studies of prion diseases, and in turn, it may provide the basis for structure-based drug design that prevents the conversion of PrP^{C} to PrP^{Sc}.

1.6 Purification methods of PrP

PrP^{C}, a membrane bound protein, which was expressed in almost mammalian species was purified from microsomal fraction by detergent extraction and separated by differential centrifugation and immobilized Cu^{2+} ion affinity chromatography. Various detergents were used to solubilize PrP^{C} from the PEG precipitates (*Table 1.5*). Pan *et al*. (1992) reported that among them, SDS and Sarkosyl give almost

Table 1.4 Cellular and scrapie PrP in hamster (Meyer et al., 1986)

Property	**PrP^{C}**	**PrP^{Sc}**
Uninfected brain	Present	Absent
Scrapie brain	Level unchanged	Accumulates
Concentration*	< 1-5 μg/g	≈ 5-10 μg/g
Purified prions	Absent	10^4 molecule per ID_{50} unit
Genetic origin	One cellular gene	One cellular gene
mRNA	2.1 kilobases	2.1 kilobases
Localization		
Intracellular	Membrane-bound	Membrane-bound
Extracellular	None	Amyloid filaments with plaque
Detergent extraction	Soluble	Amyloid rods formed
Protease digestion	Degraded	Converted to PrP 27-30+

* Expressed as μg of PrP per g brain tissue

+ PrP 27-30 is derived from PrP^{Sc} during proteinase K digestion in the absence or presence of detergent

complete solubilization of PrP^C together with many other proteins but little protein was found to bind the IMAC column in the present of either detergent. Other detergents like Triton, β-octylglucopyranoside, or ZW 3-12 do not affect PrP^C binding to the IMAC column (see ***Table 1.5***) and ZW 3-12 gave better solubilization of PrP^C than Triton and slightly higher efficiency than β-octylglucopyranoside (Pergami *et al.*, 1996). Thus ZW 3-12 was selected to use throughout this study.

In several evidences PrP^C was postulates to be a major copper-binding protein in brain. The copper ions bind with low micromolar affinity to the octapeptide repeats region in the N-terminal half of mammalian PrP^C which in mouse contains four copies of the sequence PHGG (G/S) WGQ and one copy of the sequence PQGGTWGQ (Viles *et al.*, 1999; Miura *et al.*, 1999). Spectroscopic data have suggested a bridged arrangement of coordinating histidine imidazole nitrogens binding four Cu^{2+} ions per PrP^C molecule (Martins *et al.*, 2001). In fact, copper chelate affinity chromatography has been used in the purification of PrP^C (Stockel *et al.*, 1998). Immobilized metal ion affinity chromatography (IMAC) is a purification method utilizing the interaction between biomolecules and immobilized metal ions (Porath *et al.*, 1975). In principle, some amino acid residues; histidine, tryptophan, and cysteine, can function as binding sites for metal ions. These amino acids have the electron donor atom in their side chains. Proteins or peptides containing histidine residues form more stable coordination compound with metal ions due to participation of imidazole side-chains in chelation (Sulkowski, 1985). So the presence of the clustering of histidines in the octarepeat region of PrP^C can result in strong binding of PrP^C to the IMAC column because of the cooperative interaction between histidine residues and Cu^{2+} ions (Pan *et al.*, 1992). The binding strength of PrP^C to the IMAC

Table 1.5 Solubilization of cellular prion protein (PrP^C) by detergent extraction (Pan et al., 1991)

Detergent	%Soluble[a]	Binding to IMAC[b]
SDS	>99	No
Sarkosyl	>99	No
Triton X-100	60	Yes
Triton X-114	50	Yes
β-Octylglucopyranoside	>90	Yes
ZW 3-12	>90	Yes

[a] PEG-precipitated pellets, prepared as described in the Materials and methods, were dissolved in various detergents with a weight ratio of 10 (detergent/protein). The mixtures were centrifuged at 100,000xg for 1 h. PrP^C in the pellets and supernatants was estimated by ELISA as described in Materials and methods.

[b] The IMAC-Cu^{2+} column was used to determine the binding of PrP^C to the column.

columns charged with the ions follows the order $Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$ (Porath, 1975).

Further molecular studies of prion proteins require suitable PrP separation technique, for which numerous methods have been reported in the literature. In previous purification studies, the organic extraction of PrP^C from either brain homogenate and microsomal extractions with a variety of organic solvents (such as chloroform/methanol, acetone, or ether) has been successfully applied. Although most PrP^C was precipitated out of the solution together with many other proteins, but only small amounts of PrP^C were released into the organic phase and the solubility of this precipitated PrP^C was difficult in a variety of detergent (Cambell *et al.*, 1981). In addition, Pan (1991) suggested that the method for purifying PrP accomplished by using immunoaffinity chromatography yield small amounts of protein with poor recoveries of the product.

1.7 The objectives of this thesis

A previous study (Harmeyer, 1998) using monoclonal antibodies (mAbs) generated from mouse polyclonal antibodies directed against four peptides selected from different regions of ovine PrP sequences revealed a strong reactivity with ruminant PrP^C. The mAbs that was directed against amino acid sequence 145-163 exhibited a broad cross-reactivity with immunoblotted PrP^C of different mammalian species including cattle, goat, man, pig, dog, cat, mink, and rabbit but no or weak interaction with mouse and hamster PrP^C. In this research, the isolation protocol does not require an immunoaffinity purification step as utilized in the past. The procedure for partial purification of PrP in this study includes differential centrifugation and

detergent extraction. A crude microsomal fraction from animal brains was obtained by low-speed centrifugation followed by precipitation with polyethylene glycol (PEG)-8000. PrP^C was solubilized from this membrane fraction with Zwittergent (ZW) 3-12 and centrifuged at 100,000 xg. Fractionation was then applied onto an immobilized metal-ion affinity chromatography (IMAC) column that had been charged with Cu^{2+}. The resulting fractions were further purified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The aim of this thesis is the generation of polyclonal antibody directed against synthetic peptides for detection of cellular prion protein (PrP^C) in economic animal of Thailand for further understanding of the prions at the molecularlevel.

The aim of this thesis as follows:

1. To generate polyclonal antisera against synthetic peptide corresponding to amino acids sequences in some region of sheep prion protein
2. To extract and partially purify cellular prion proteins from animal brains: fish, pig, and cow
3. To detect the extracted cellular prion proteins from brain tissues by using generated polyclonal antisera

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments and Materials

Affinity columns: HiTrap^{Tm} Chelating HP 1 ml (17-0409-01), Amersham Pharmacia Biotech Inc., U.S.A.

Analytical Balance: - Satorius BP 310S, Scientific Promotion Co., Ltd., Germany
- Mettler AB 204-S, Mettler-Toledo, Switzerland

Autopipette: - Pipetman, Gilson, France
- Multichannel pipette, High Tech Labs, Poland

Centrifugal filter devices: Microcon YM-10, molecular weight limit 10 kDa, Millipore Corporation, U.SA.

Centrifuge: - Refrigerated centrifuge, Coulter Avanti J-30I Beckman, Beckman Instrument Inc., U.S.A.
- Microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan
- Ultracentrifuge: Model L8-70 Beckman, Beckman Instrument Inc., U.S.A.

Electroblotting apparatus: Mini Compact Electro-Blotting unit, Vertical, U.K.

Electrophoresis apparatus: Mini Protean®3 System, BIO-RAD, U.S.A.

ELISA microtiter plate 96 wells: Corning, U.S.A.

ELISA microtiter plate reader: Titertek Multiscan Plus, Labsystems Ins., Findland

FPLC: AKTA: Amersham Pharmacia Biotech Inc., U.S.A.

Homogenizer and glass tube: Glas-Col, U.S.A.

Nitrocellulose membrane: Hybond^{TM} ELC^{TM}, Amersham Pharmacia Biotech Inc., U.S.A.

pH meter: Model : PHM 83 Autocal pH meter, Radiometer, Denmark

Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.

Spectrophotometer: - Spectronic 2000, Bausch & Lomb, U.S.A.

- UV-240, Shimadzu, Japan

Syringe and 21-g needle: Nipro Tuberculin, Nissho Nipro Corp., Ltd., Thailand

Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.

2.2 Chemicals

Acrylamide (17-11302-01): Pharmacia Biotech, Sweden

Ammonium persulphate (A-3678): Sigma, U.S.A.

β- mercaptoethanol (63690): Fluka, Switzerland

Bovine serum albumin (05480): Fluka, Switzerland.

Complete Freund's adjuvant (F-5881): Sigma, U.S.A.

Coomassie brilliant blue R-250 (B-0149): Sigma, U.S.A.

Copper sulfate: Merck, Germany

3,3'-Diaminobenzidine (DAB) (32750): Fluka, Switzerland

Ethylene diamine tetraacetic acid (EDTA) (03685): Fluka, Switzerland

Horseradish peroxidase-conjugated goat antibody to mouse IgG : Sigma, U.S.A.

Imidazole (56750): Fluka, Switzerland

Incomplete Freund's adjuvant (F-5506): Sigma, U.S.A.

N-Dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (ZW 3-12) (D-0431): Sigma, U.S.A.

N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (T-4376): Sigma, U.S.A.

N,*N*'-methylene-bis-acrylamide (M-7256): Sigma, U.S.A.

N,*N*,*N*',*N*'-tetramethyl-1, 2-diaminoethane (TEMED) (T-8133) Carlo Erba Reagenti, Italy

Phenylmethylsulfonyl fluoride (PMSF) (20203): Amersham Pharmacia Biotech Inc., U.S.A.

Phosephate buffered saline, pH 7.5 (PBS) (P-4471): Sigma, U.S.A.

Polyethyleneglycol-8000 (PEG-8000) (19959): Amersham Pharmacia Biotech Inc., U.S.A.

Prestained protein ladder (SM-0671): Fermentas Inc.,U.S.A.

Sodium dodecyl sulfate (SDS) (L-5750): Sigma, U.S.A.

Sucrose (84105): Fluka, Switzerland

3,3',5,5'-Tetramethylbenzidene (TMB) (T-2885): Sigma, U.S.A.

Tris (hydroxymethyl)-aminomethane (77-86-1): Carlo Erba Reagenti, Italy

Tween 20 (93773): Fluka, Switzerland

Urea hydrogen peroxide (U-1753): Sigma, U.S.A.

Other common chemicals were obtained from Fluka, Sigma, Carlo or BDH. All chemicals are of reagent grade unless otherwise specified. All water used was distilled once on an automatic water distiller.

2.3 Animals

Nine female BALB/cA mice (inbred type) were purchased from National Laboratory Animal Center Mahidol University (NLAC-MU), Nakornpatom. The mice are about 6 to 8 weeks of age. The animals were maintained in the Laboratory animals' house of the Department of Biology, Faculty of Science according to the

local guidelines for animal welfare. The mice were allowed resting for 1 week before the primary immunization.

2.4 Brain Samples

Brain samples were collected from animals found in local markets; catfish brain of from Klong Tuey market, cross-bred pig brain from Phran Nok market, and Bos Inbicus cow brain from a market in Kalasin Province, northeast of Thailand, 519 km from Bangkok. These samples were removed from the animals as soon as possible post mortal, transported on ice to our laboratory, collected in ice, and stored at -80^{o} C until use.

2.5 PrP antibodies

Monoclonal antibody L42 used in this study was purchased from R-Biopharm AG, Germany. The mAb L42 was directed against an epitope region of amino acids sequence 145 to 163 of sheep PrP. This antibody is described in detailed elsewhere (Harmeyer *et al.*, 1998).

Mouse polyclonal antisera generated in laboratory for this study were raised in BALB/cA mice against 3 synthetic peptides corresponding to sheep PrP amino acids sequence as described in section 2.6; PI (40-56), PII (145-163), and PIII (209-228) (Groschup *et al.*, 1997; Harmeyer *et al*., 1998).

2.6 Production of synthetic peptides

Three synthetic peptides corresponding to amino acids sequences in three different regions of sheep PrP; PI; 40 to 56 (RYPGQGSPGGNRYPPQG), PII; 145 to

163 (GNDYEDRYYRENMYRYPNQ) and PIII; 209 to 228 (MERVVEQMCITQ YQRESQAY) (Groschup *et al.*, 1997; Harmeyer *et al.*, 1998) were custom-synthesized by Sigma-Genosys of Sigma Aldrich Co., U.S.A. using an automated peptide synthesizer and MAP (multiple antigenic peptide) system (Tam, 1988; Tam *et al.*, 1989). In this procedure, peptides were generated on four-branch 9-fluorenyl methyl-oxycarbonyl (Fmoc) MAP resin (***Figure 2.1 and 2.2***). These obtained peptides are white powder with the yield of 27.1 mg, 14 mg, and 15.9 mg of P I, P II, P III, respectively.

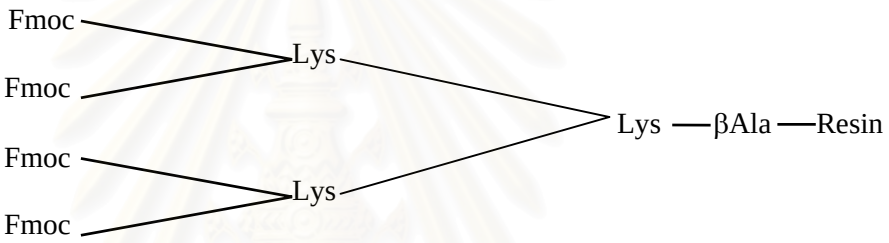

Figure 2.1 Fmoc MAP Resins 4-Branch (Tam, 1988)

2.7 Immunization with synthetic peptides

Three MAP-peptides were emulsified in Complete Freund's adjuvant (CFA) for the first immunization and in Incomplete Freund's adjuvant (IFA) for all subsequent immunizations. For each peptide antigen, three female BALB/cA mice were immunized subcutaneously at multiple sites according to a published schedule (Harmeyer *et al.*, 1998). The primary immunization contained 200 μg for each of the MAP-peptide and each booster injection contained 100 μg. Booster injections were given five times for five weeks. Blood samples were taken from the heart 1 week after the last booster injection. After that, the blood was allowed to clot at room temperature for about 1 h then left overnight at 4°C, centrifuged at 2,500 rpm for 30

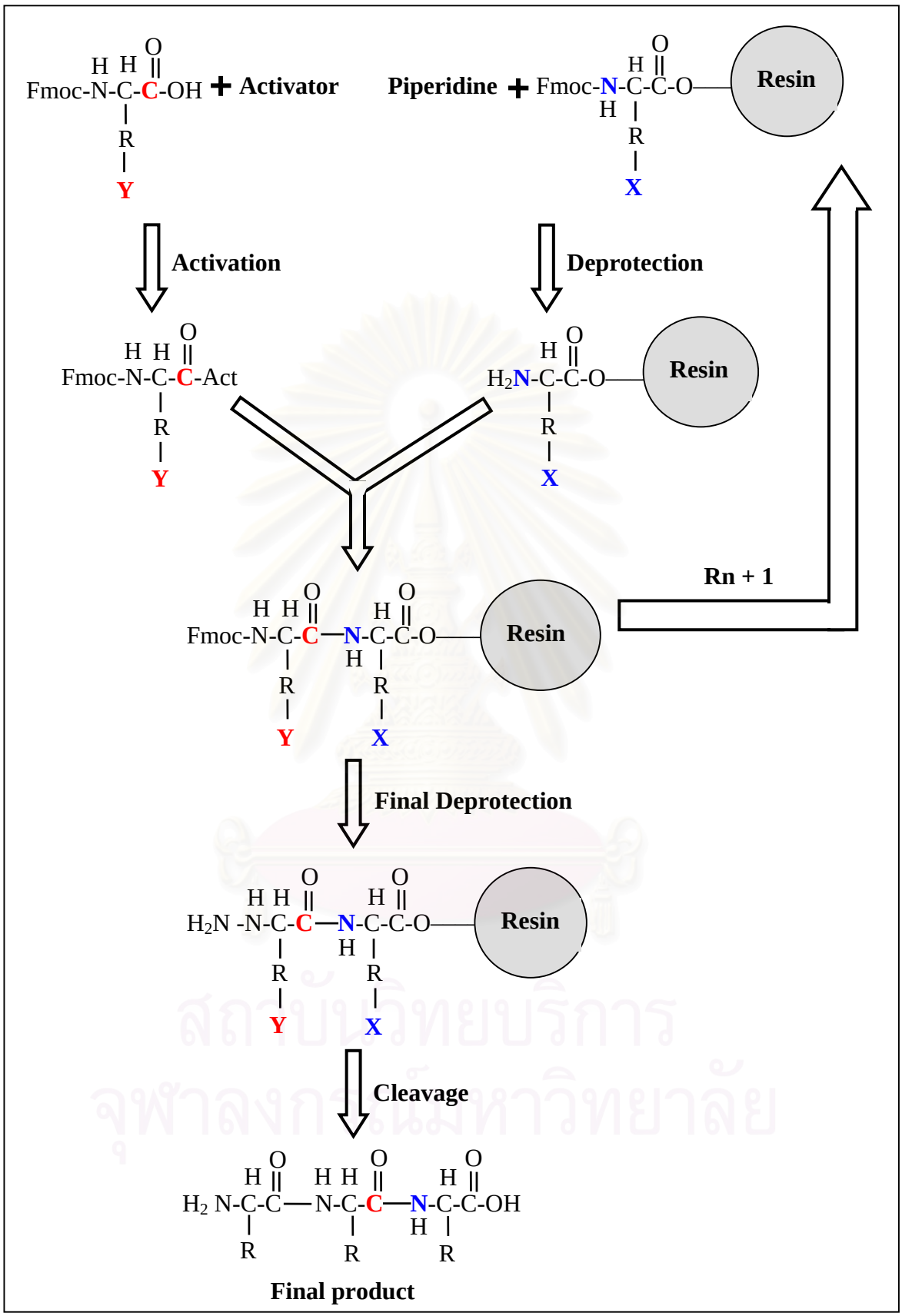

Figure 2.2 Basic steps in Solid Phase Peptide Synthesis using Fmoc chemistry

(http://www.sigma-genosys.com/media/Solid_Phase_Synthesis.pdf)

min at 4°C and tested in an enzyme-linked immunosorbent assay (ELISA) to determine the antibody titers (OD_{50} values) as described in section 2.11. The mouse polyclonal antisera were stored at -20°C for further applications.

The blood of non-immunized mouse was collected as the non-immune antiserum.

2.8 Extraction and partial purification of cellular PrP

Cellular PrP was extracted from animal brains of pig, fish, and cow and purified by immobilized metal affinity chromatography (IMAC) using published procedures (Pan *et al*., 1992). Briefly, the animal brain tissue was homogenized with an electric homogenizer in 6 volume (v/w) of 0.25 M sucrose, 0.1 mg/ml *N*-tosyl-L-phenyl alanine chloromethyl ketone (TPCK, a chymotrypsin-like serine protease inhibitor), 1 mM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), 10 mM sodium phosphate buffer (pH 7.0), and 0.15 M NaCl. Cell debris was spun down by centrifugation at 3000 xg for 30 min. After that the supernatant was collected. Then proteins were precipitated by an addition of PEG-8000 to make a final concentration of 4% (w/v). The suspension was stirred at 4°C for 15 min and centrifuged at 14,000xg for 10 min. The precipitates were solubilized in a solution of 0.15 M NaCl, 10 mM sodium phosphate buffer (pH 7.0), 1 mM PMSF, 0.1 mg/ml TPCK, and 8% (w/v) ZW 3-12. The mixture was stirred at 4°C for 1 h and then centrifuged at 100,000xg at 4°C for 1 h. The supernatant of microsomal fraction was filtered through sterilized filter paper (0.45 μm pore size) for further partially purified by IMAC. The final supernatant was applied onto a chelating Sepharose Fast Flow column (HiTrap affinity column, 1 ml), which was charged with Cu^{2+} ions and

equilibrated in 0.2% ZW 3-12, 10 mM sodium phosphate buffer (pH 7.0), and 0.15 M NaCl (starting buffer). The column was then washed with 10 mM imidazole in starting buffer (buffer I), and followed by 0.2% ZW 3-12, 0.1 M sodium acetate buffer (pH 5.0), and 0.5M NaCl (buffer A). Finally, bound PrP^C was eluted with 50 mM ethylene diamine tetraacetic acid (EDTA), 0.2% ZW 3-12, 10 mM sodium phosphate buffer (pH 7.0), and 0.2 M NaCl (buffer E). The flow rate was 1 ml/min and 3-ml fractions were collected. The absorbance of each fraction was measured at 280 nm. The eluate was stored at -20^{o}C for the further applications. The steps of extraction and partial purification are summarized as shown in ***Figure 2.3***.

2.9 Protein determination

Protein concentrations were determined by the method of Bradford (1976). Distilled water was added to a protein solution (maximum 100 µl) to make a total volume of 100 µl. Then 1 ml of Bradford working buffer was added and mixed. After 10 minutes but before 1 hour, A_{595} was recorded. Bovine serum albumin was used as standard protein. The standard curve is shown in Appendix 4.

2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed by discontinuous SDS-PAGE in a denaturing condition according to Laemmli (1970). Each was electrophoresed onto a 12% polyacrylamide gel. Resolved proteins on the gel were visualized by Coomassie Blue staining and eletrophoretically transfer onto a nitrocellulose membrane for immunoblotting as described in section 2.12.

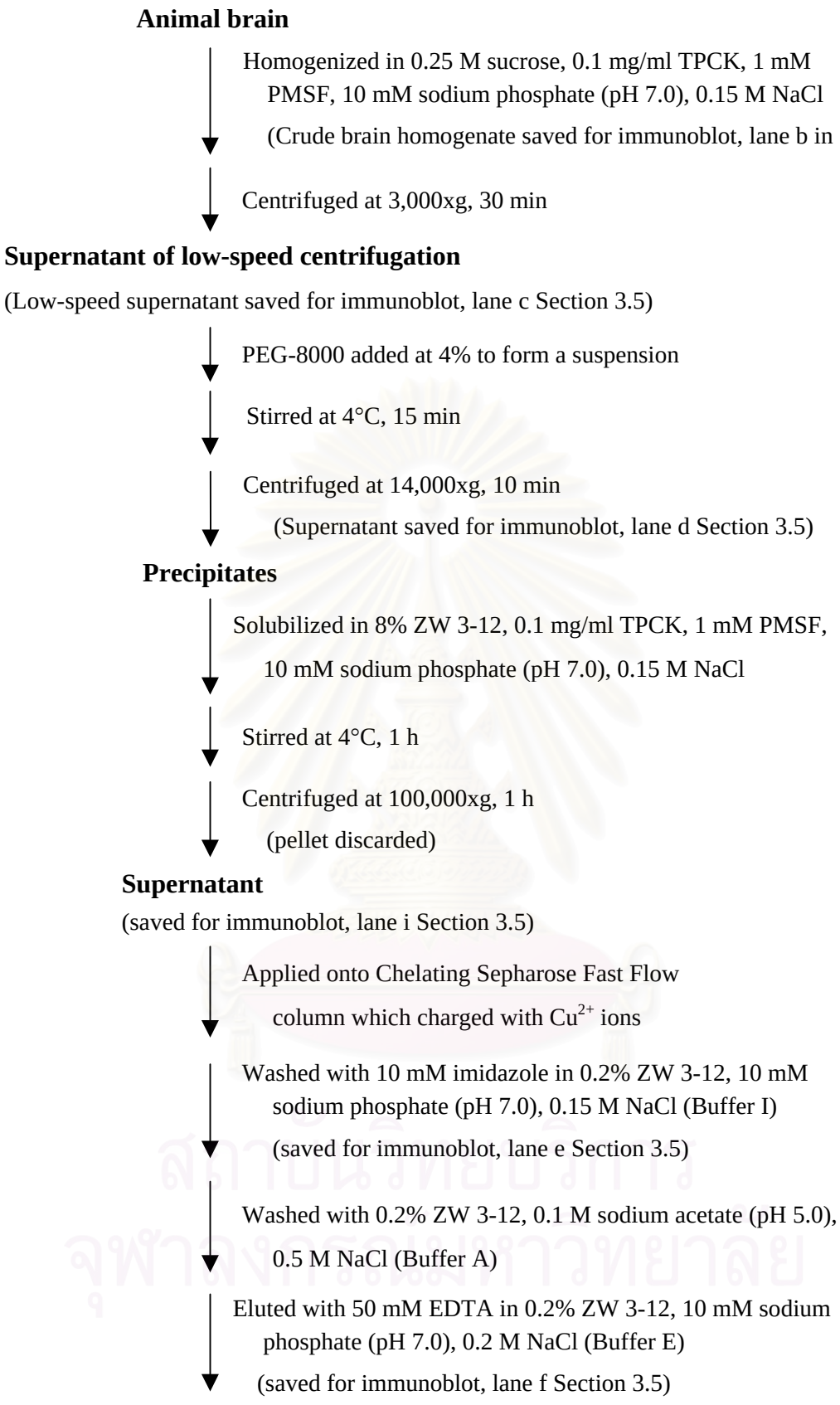

Figure 2.3 Flow chart of PrP extraction and partial purification, summarizing a published procedure (Pan et al., 1992)

2.10.1 Preparation of the separating gel (12% acrylamide)

For the separating gel (10 x 8 x 0.1 cm), the 3.0 ml of solution A (30% acrylamide and 0.8% bis-acrylamide) was mixed with 2.5 ml of solution B (2 M Tris-HCl, pH 8.8 and 10% SDS) and 1.89 ml of distilled water (see Appendix 1). Then 50 μl of 10% ammonium persulfate and 10 μl of TEMED were added and mixed rapidly. The mixture was carefully introduced in between the two glass plates of a gel casting frame using an autopipette. After the appropriate amount of separating gel solution was added, the water was gently layered about 1 cm height on top of the separating gel solution. For at least three hour, the gel was allowed to polymerize as indicated by a clear interface between the separating gel and the water. The water was then poured off.

2.10.2 Preparation of the stacking gel (4 % acrylamide)

For the stacking gel (10 x 2 x 0.1), 0.67 ml of solution A was mixed with 1.0 ml of solution C (1 M Tris-HCl, pH 8.8 and 10% SDS) and 3.27 ml of distilled water (see Appendix 1). Subsequently, 50 μl of 10% ammonium persulfate and 10 μl of TEMED were added and mixed rapidly. This stacking gel solution was loaded onto the separating gel until the solution reached top of the short glass plate. Then the Teflon comb was carefully inserted into the gel. After the stacking gel appeared to been completely polymerized, the comb was carefully removed. Then the gel was placed into the electrophoresis chamber. The electrophoresis buffer (see Appendix 1) was added into the reservoir. Air bubbles present in the well were removed.

2.10.3 Sample preparation

The protein sample was mixed with sample buffer (see Appendix 1). Prior to the application, each was denatured by boiling at 100^{o}C for 5 min. Then the 20 μl of the sample solution was introduced into well by using a microsyringe.

2.10.4 Running the gel

The electrophoresis was performed from the cathode towards the anode, at a constant current of 20 mA per slab, at room temperature in a Mini Protean®3 System Gel Electrophoresis unit and continued until the dye front reached the bottom of the gel. Power was turned off and the electrode plugs were then removed from the electrodes. The gel plates were, in turn, removed from the electrophoresis chamber. Then the gel was removed from glass plates and transferred to a small container. The standard molecular weight marker (Prestained Protein Ladder, 10-180 kDa) was a mixture of 10 recombinant, highly purified color proteins. Following electrophoresis, the gel was stained for proteins as described in section 2.10.5 or electrotransferred immediately to nitrocellulose as described in section 2.12 without staining the gel.

2.10.5 Detection of protein

Coomassie Blue staining

The gel from 2.10.4 was transferred to a small container containing Coomassie staining solution (see Appendix 1). The gel was agitated for 10-20 minutes on a shaker. The staining solution was poured out and the destaining solution

was added. The gel was shaken slowly in 2-3 changes of the destaining solution and agitated overnight or until blue bands of protein were visible.

2.11 Enzyme-linked Immunosorbent Assay (ELISA)

The binding of antibody to synthetic peptides antigens was determined by Indirect Enzyme-linked Immunosorbent Assay (indirect ELISA). In this study, the ELISA assay was modified from the general protocol and the method of Harmeyer *et al.* (1998) as illustrated in ***Figure 2.4***. Synthetic peptides were first solubilized in 0.1 M sodium carbonate buffer (pH 9.6) to a final concentration of 0.2 μg per well. A 50 μl aliqout of peptide antigen was pipetted onto each of the 96-well onto ELISA microtiter plates (Corning, U.S.A.) and coated overnight at room temperature. Unbound antigens were removed by washing the plate three times with 300 μl of PBS buffer containing 0.1% (v/v) Tween 20. The washing solutions were removed by inverting the plate quickly and tapping the plate on a paper towel to remove any drops. Non-specific binding sites in each well were blocked with 300 μl of blocking solution (3% (w/v) BSA in PBS-Tween 20). The plate was incubated for 1 h at room temperature and then washed three times with 300 μl of PBS-Tween 20. Mouse polyclonal antisera and pre-immune mouse antisera were prepared by making two-fold serial dilutions (ranging in dilution from 1:12.5, 1:25, 1:50…1:3,355,443,200) in blocking solution. A 50 μl aliqout of each dilution was added onto an antigen-coated well. The plate was then incubated for 2 h at room temperature and washed four times with 300 μl of PBS-Tween 20 to remove the unbound antisera. After that the plate was added with 50 μl of Horseradish peroxidase-conjugated goat antibody

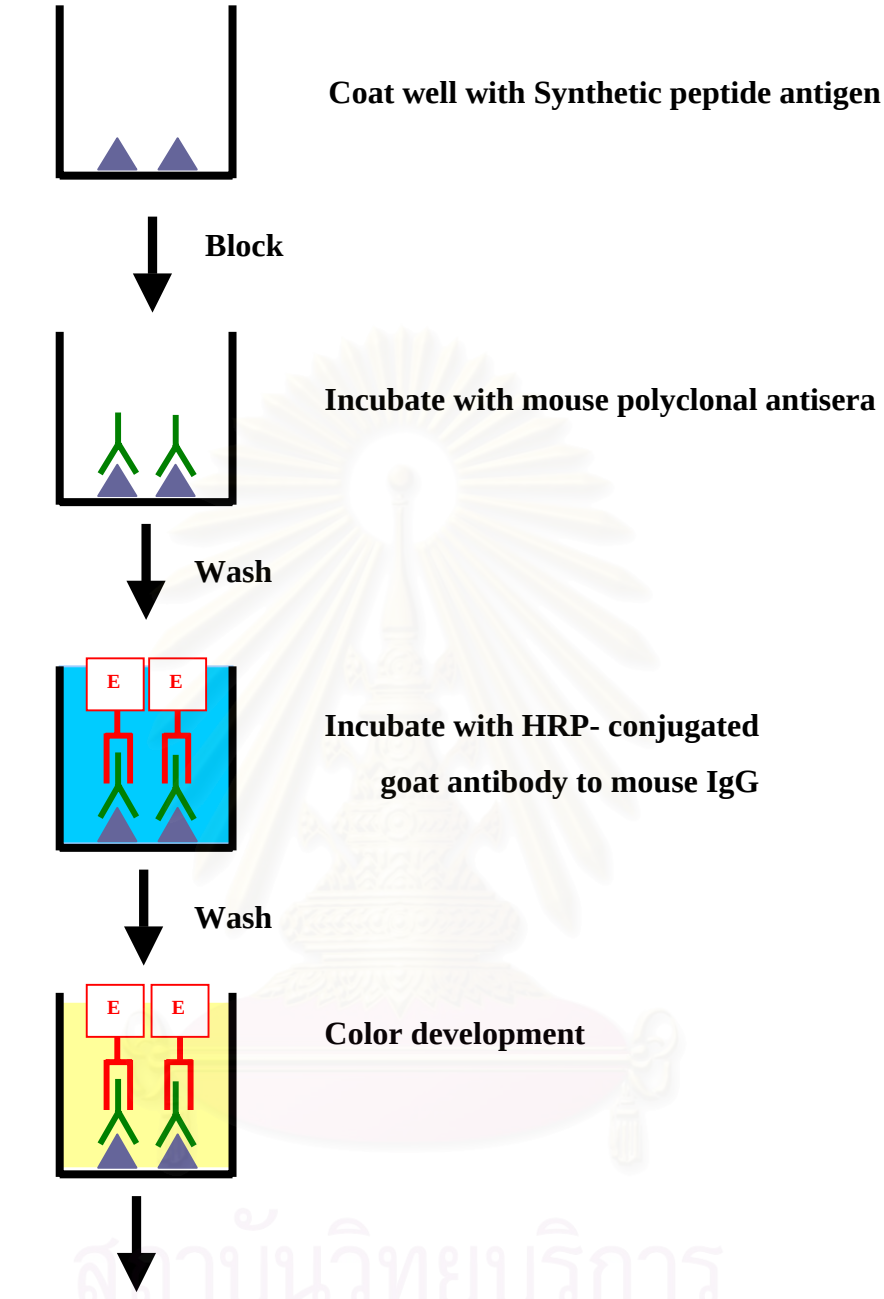

Figure 2.4 Illustration of indirect ELISA for determination of antibody titer (OD_{50} values) of mouse polyclonal antisera.

= Synthetic peptide antigens, = mouse polyclonal antisera

E

= Horseradish peroxidase-conjugated goat antibody to mouse IgG

against mouse IgG at 1:1000 dilution in blocking solution and incubated for 2 h at 37° C and then washed four times with 300 μl of PBS-Tween 20. The substrate solution was prepared just before use by dissolving 0.1 mg of 3,3',5,5'-Tetramethylbenzidine (TMB) in 100 μl of dimethylsulfoxide, then adding in 9.9 ml of 0.1 M sodium acetate buffer, pH 5.0 and finally adding 0.1 mg urea hydrogen peroxide as a substrate. The enzymatic activity was determined by adding 100 μl of substrate solution to each well so that a blue product would form. The reaction was stopped after 5 min by the adding of 50 μl of 2 M H_2SO_4 that caused the product changed to yellow color. The absorbance at 450 nm of each well was read in an ELISA microtiter plate reader. Antibody titers (OD_{50}) of mouse polyclonal antisera were determined by finding the antibody dilution giving 50% of the absorbance of the maximal color reaction.

2.12 Immunoblotting Assay

Following electrophoresis by SDS-polyacrylamide gels using the method as described in section 2.10, the gel as well as the nitrocellulose membrane were incubated for 10-15 min in tank-blotting transfer buffer (see Appendix 2) prior to blotting. Sheets of material were placed up against one another in this order while avoiding air bubble: 4 sheets of filter paper on the fiber pad, the gel, the membrane, 4 sheets of filter paper, and finally the other fiber pad. The installed Mini Compact Electro-blotting unit was filled with approximately 1.5 l of the tank-blotting transfer buffer. The separated proteins on the gel were then electrophoretically transferred onto the nitrocellulose membrane from the cathode towards the anode at 50 volts for 2.5 h. During the transfer, the system was cooled down to 4°C in an ice bucket. After the transfer, the orientation of the gel was marked on the membrane. The

membrane was washed twice for 10 min each time with TBS buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl) at room temperature. The membrane was then incubated in the blocking solution (3% (w/v) BSA in TBS buffer) at 37°C for 1 h. After that, the membrane was washed twice for 10 min each time in TBS containing 0.025% (v/v) Tween 20 and rinsed 10 min with TBS buffer at room temperature. Subsequently, the membrane was incubated for 1 h at 37°C in the blocking solution containing either mAb L42 (at 1:1000 dilution) or mouse polyclonal antisera (at 1:150 dilution from section 2.6). The membrane was then washed twice for 10 min each time in TBS-Tween 20 buffer and rinsed for 10 min in TBS buffer at room temperature. The membrane was finally incubated with Horseradish peroxidase-conjugated goat antibody against mouse IgG at 1:1000 dilution in blocking solution for 1 h at 37°C, and then washed four times for 10 min each time in TBS-Tween 20 at room temperature. After washings, immunoreactive staining was developed while keeping the membrane stationary with 3,3'-Diaminobenzidine (DAB) and 30% hydrogen-peroxide until the bands were clearly visible (approximately 10 min). The chromogenic reaction was stop by rinsing the membrane twice with distilled water.

CHAPTER III

RESULTS

3.1 Production of synthetic peptides

Three immunogenic, prion fragments were custom-synthesized by Sigma-Genosys of Sigma Aldrich Co., U.S.A. using an automated peptide synthesizer and multiple antigenic peptide (MAP) system, which has been reported to give stronger immune response compared to a monomeric peptide. The system is based on a small inert branched lysine core onto which the multiple peptides are synthesized in parallel by using standard solid phase peptide synthesis. Each core contains four copies of identical peptides giving a high molar ratio of peptide antigen to core molecule. After synthesis, the obtained peptide does not require the use of a carrier protein such as keyhole limpet hemacyanin (KLH) or bovine serum albumin (BSA) for conjugation in order to induce antibody response. These synthetic peptides were generated on four-branch 9-fluorenylmethyl-oxycabonyl (Fmoc) using solid phase peptide synthesis (SPPS) as described in ***Figure 2.2***. In this study three synthetic peptides were synthesized according to amino acids sequences in three regions of published sheep prion protein as shown in ***Table 3.1*** these sequences have been reported to show strong antibody bindings under ELISA and good reactivity with prion proteins under immunoblot (Harmeyer *et al.*, 1998). Moreover the amino acid sequences 145-163 have been shown to cross-react among prion protein of mammalian species (Groschup *et al.*, 1997; Harmeyer *et al.*, 1998). These synthetic peptides were used for immunizing mice in the production of the antisera used for further detection of prion proteins in animal brain tissue extracts.

Table 3.1 Amino acid sequence of synthetic peptides

Synthetic peptides	Position	Length (a.a. residues)	Amino acid sequence
P I	40-56	17	RYPGQGSPGGNRYPPQG
P II	145-163	19	GNDYEDRYYRENMYRYPNQ
P III	209-228	20	MERVVEQMCITQYQRESQAY

Note: In order to demonstrate the sequence accuracy of peptide synthesis, the synthetic peptide II (PII) was subjected to N-terminal peptide sequencing at the Bio Service Unit (BSU), National Science and Technology Development Agency. This resulting sequence, shown in Appendix 7, synthesis specification given by Sigma-Genosys of Sigma Aldrich Co., U.S.A.

3.2 Generation and characterization of polyclonal antisera in mice.

In previous studies, each of 16 different prion fragments spanning to the full-length amino acid sequence of ovine PrP, including these three synthetic peptide fragments used in this study were used to immunize chicken and rabbit by Groschup *et al.* (1997), and mice by Harmeyer *et al.* (1998). In this study, nine BALB/cA mice were divided into 3 groups, each group was separately immunized with synthetic peptides PI, PII, and PIII. The primary immunization contained 200 μg of the MAPs-peptide emusified in Complete Freund's adjuvant and all boost injections contained 100 μg emusified in Incomplete Freund's adjuvant. Boost injections were given five times in 5 weeks. One week after the last boost injection, blood samples were taken from the animals' hearts, and then centrifuged to collect the serum. The antisera were tested in an enzyme-linked immunosorbent assay (ELISA) to determine of the antibody titers (OD_{50} values), on microtiter plates coated with corresponding syntheic peptides. The antiserum elicited by each peptide displayed antibody binding to the homologous peptide (***Figure 3.1***). As shown in ***Table 3.2*** all immunized mice with each peptide displayed antibody titers (OD_{50} values) against sequences 40 to 56 (Ab PI), 145 to 163 (Ab PII), and 209 to 228 (Ab PIII) at dilutions of 1:1,600, 1: 51,200 and 1:1,638,400, respectively. Non-immune sera showed weaker reactions with PI and PII, and almost no reaction with PIII under ELISA. These results could be observed even by naked eyes.

Originally, the polyclonal antiserum that showed the highest titer in each group (Ab PI, PII, or PIII) was to select for subsequent identification of extracted PrP by immunoblot. As it turned out (***Table 3.2***), the titers of all antisera in each group were equal. Subsequent experiments involving immunoblots were therefore carried out using antisera from mouse No. 1 in each group.

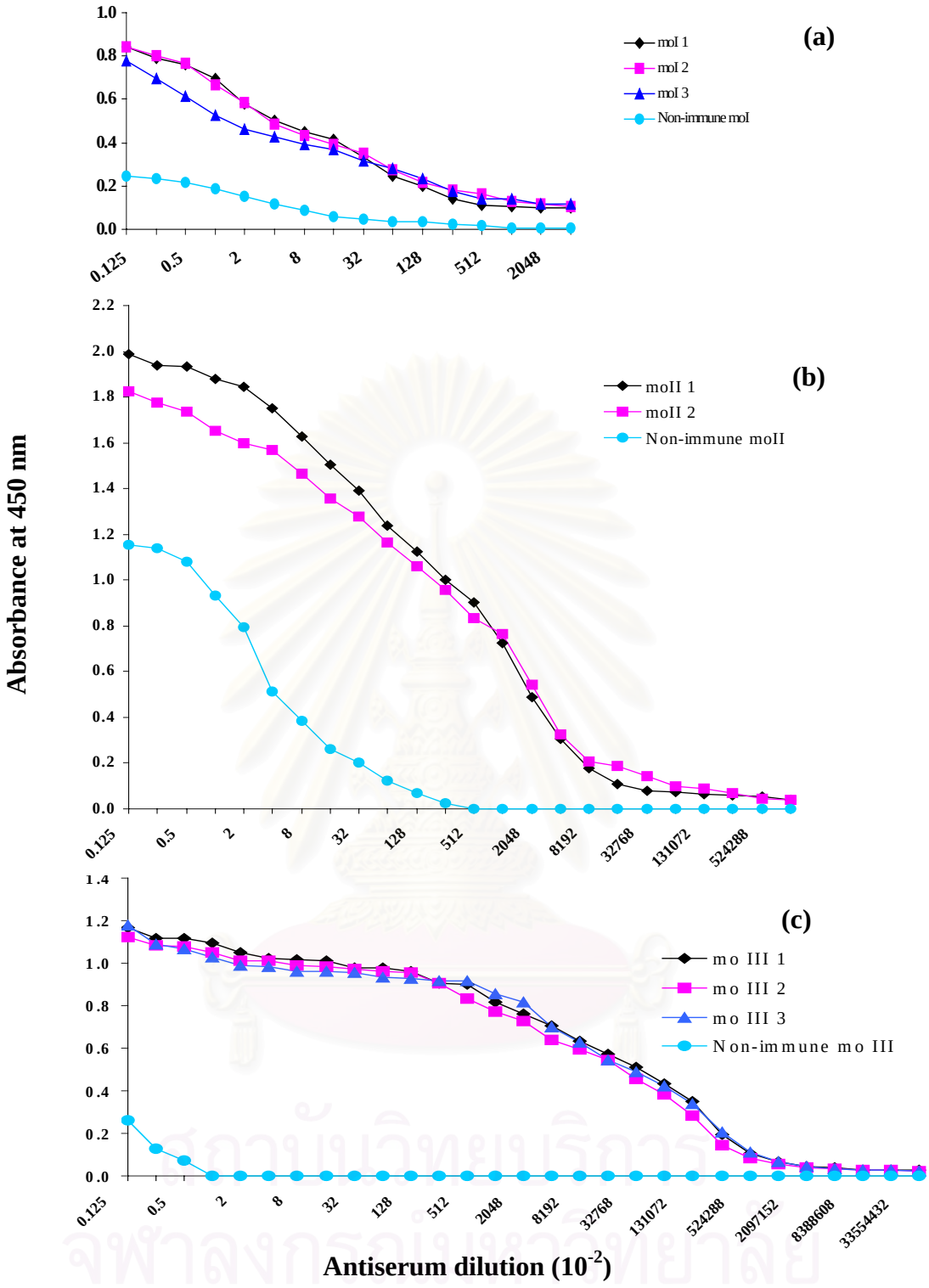

Figure 3.1 Reactivity of mouse polyclonal antisera against synthetic peptide antigens. Each group has been linearly consolidated from two or more graphs each covering a different range of dilution. (a) antisera against 40-56 (Ab PI), (b) antisera against 145-163 (Ab PII) and (c) antisera against 209-228 (Ab PIII) in an ELISA.

Table 3.2 Antibody titers (OD_{50} values) of 9 mice polyclonal antisera

Antisera against synthetic peptide antigens (amino acid residues)	Mouse	Antibody titers (OD_{50})**
PI (40-56)	moI 1	1:1,600
	moI 2	1:1,600
	moI 3	1:1,600
PII (145-163)	moII 1	1:51,200
	moII 2	1:51,200
	moII 3*	—
PIII (209-228)	moIII 1	1:1,638,400
	moIII 2	1:1,638,400
	moIII 3	1:1,638,400

* moII 3 died during the first week of immunization period.

** Antibody titers of the antisera were calculated as reciprocal antiserum dilutions giving 50% immunostaining in an Enzyme-linked immunosorbent assay (ELISA) using the corresponding synthetic peptides as antigens.

3.3 Partial purification of cellular prion protein (PrP^C)

In this study, cellular PrP was partially purified according to the method described in Section 2.8. The PrP^C was purified from microsomal fraction by detergent extraction and separated by differential centrifugation and immobilized Cu^{2+} ion affinity chromatography. The animal brain homogenate was first centrifuged at 3,000 xg and then precipitated by PEG-8000. The precipitates were solubilized in 8% ZW 3-12 and centrifuged to remove insoluble material. After high-speed centrifugation PrP^C became soluble in the supernatant of ZW 3-12 extraction. Total protein in each purification step was determined by the method of Bradford (1976) as shown in ***Table 3.3***. ***Figure 3.2 a***, ***b***, and ***c*** show the elution profile of ZW 3-12 solubilized microsomal fraction on an IMAC-Cu^{2+} column from fish, pig, and cow brain extract, respectively. Although other proteins were eluted from the column by washing with acetate buffer, pH 5.0 (Arrow A), the lowing of pH did not affect the binding of PrP^C (Harmeyer *et al.*, 1998). PrP^C was eluted by either imidazole (Arrow I) or EDTA (Arrow E). SDS-PAGE revealed that PrP^C was indeed partially purified by immobilized Cu^{2+} ion affinity chromatography because fewer protein bands were observed in the EDTA eluate fraction of the IMAC column as shown in Section 3.5.

Table 3.3 Total protein yields at various steps of prion purification.

Steps	Total protein in brain tissue (mg/g brain)		
	Fish	**Pig**	**Cow**
Brain homogenate	47.19	17.95	19.75
Supernatant of low-speed centrifugation	20.59	16.22	14.37
Supernatant of PEG-8000 Precipitation	15.04	10.45	12.92
Supernatant of ZW 3-12 Extraction	2.64	0.99	0.44
EDTA eluate of IMAC Column	0.0076	0.0087	0.0022

Cellular PrP were solubilized from the microsomal fraction by the detergent, ZW 3-12, and partially purified by differential centrifugation and immobilized metal ion affinity chromatography (IMAC) from animal brain tissues as described in Section 2.8. Total proteins were determined by the method of Bradford as described in Section 2.9. Fish brain (42.2 g) was collected from 68 fish. Pig brain (103 g) was obtained from 1 whole pig brain. Cow brain (224 g) came from the right lobe after sagittal sectioning of a whole cow brain weighing 365 g.

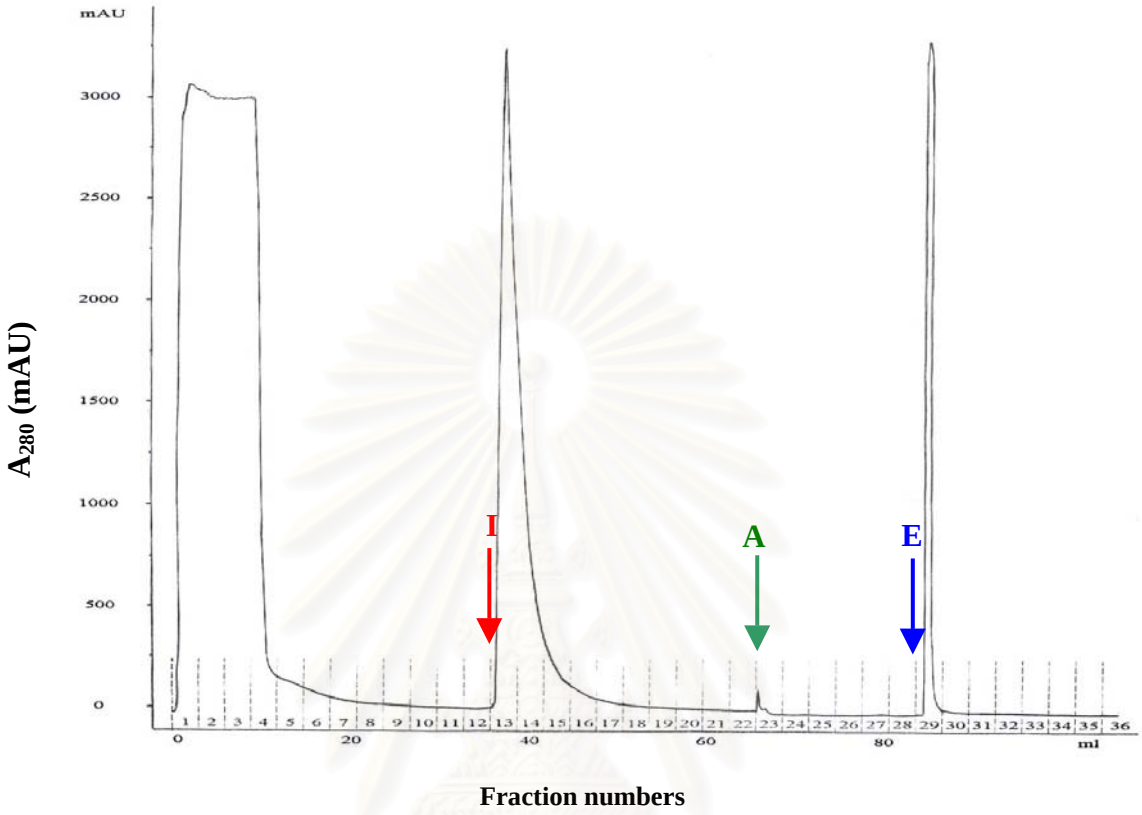

Figure 3.2(a) Immobilized metal-ion affinity chromatography (IMAC) profile of ZW 3-12 solubilized microsomal fraction from fish brain extraction.

The supernatant of microsomal fraction was applied onto an IMAC column (1 ml) charged with Cu^{2+} ions, which was previously equilibrated with starting buffer (Section 2.8). The flow rate was 1 ml/min, and 3-ml fractions were collected. Arrow I marks the start of 10 mM imidazole in phosphate buffer, pH 7.0 (Buffer I) wash. Arrow A marks the acetate buffer, pH 5.0 (Buffer A) wash. Arrow E marks elution of PrP by 50 mM EDTA in phosphate buffer, pH 7.0 (Buffer E).

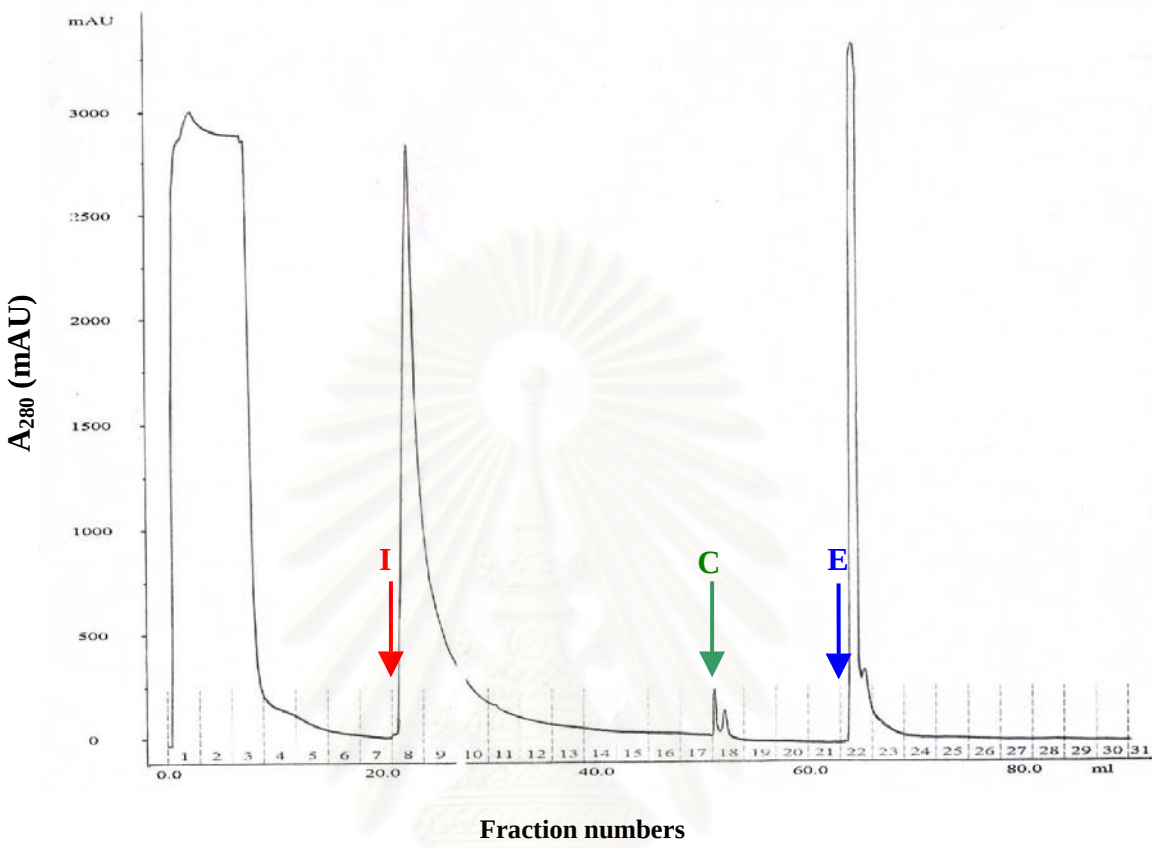

Figure 3.2(b) Immobilized metal-ion affinity chromatography (IMAC) profile of ZW 3-12 solubilized microsomal fraction from pig brain extraction.

Arrow and labeled in the same fashion as in Figure 3.2(a).

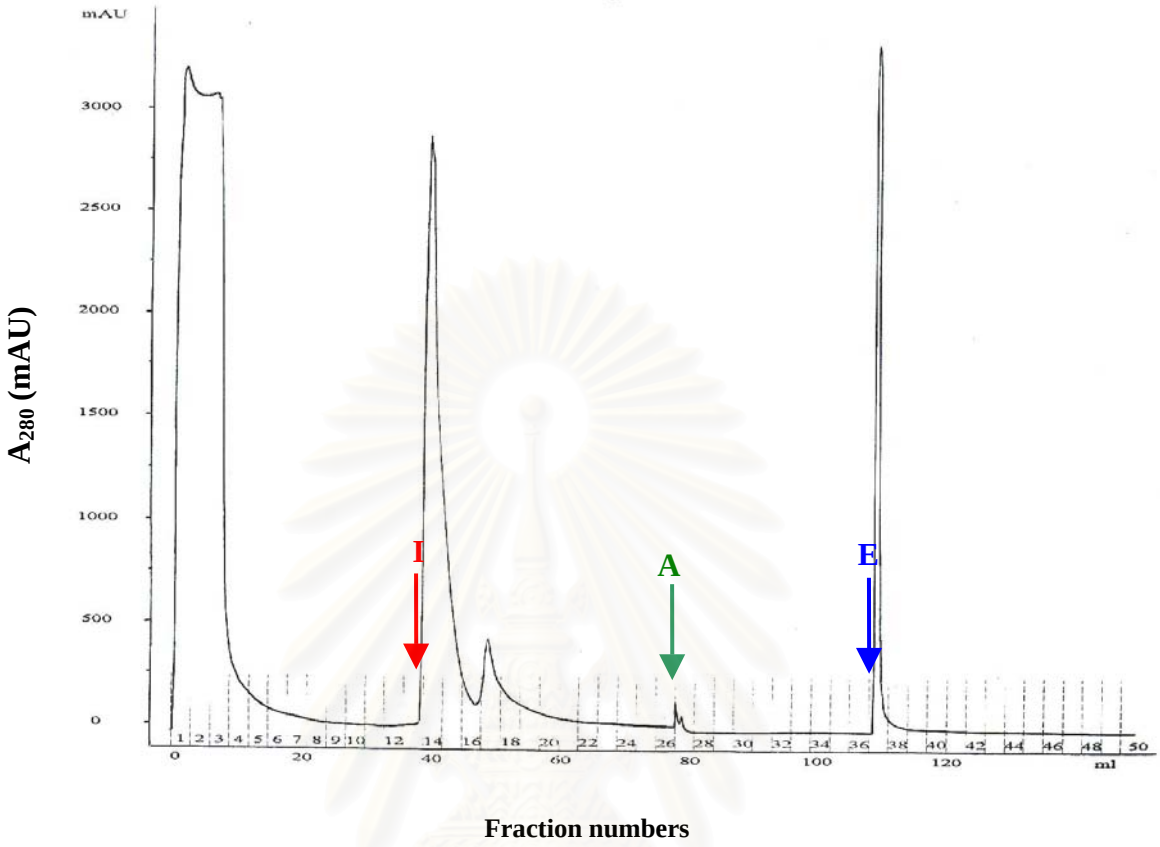

Figure 3.2(c) Immobilized metal-ion affinity chromatography (IMAC) profile of ZW 3-12 solubilized microsomal fraction from cow brain extraction.

Arrow and labeled in the same fashion as in Figure 3.2(a).

3.4 Reactivity of mouse polyclonal antisera with cellular PrP (PrP^C) in crude brain homogenates

Each mouse polyclonal antisera; Ab PI (40-56), Ab PII (145-163), and Ab PIII (209-228), was subsequently tested for their ability to detect PrP^C from fish, pig, and cow brain. PrP^C could be detected in crude brain homogenates with Ab PI, Ab PII, and Ab PIII giving the observed bands on nitrocellulose membrane, using the corresponding synthetic peptide as a positive control for each polyclonal antisera. The results indicated that in PrP prepared from crude brain homogenates of pig and cow, the obvious protein band at the molecular mass (M_r) of 40 kDa could be observed on the nitrocellulose membrane. In addition, the other major band of approximate M_r 30 kDa which was visible in pig and especially in cow (***Figure 3.3*** lane c and lane d, respectively) was not observe in crude brain homogenates of fish in immunoblot (***Figure 3.3*** lane b). A similar result was obtained using a reference monoclonal antibody; mAb L42 (see ***Figure 3.3 d***) which was raised in mice directed against amino acid residues 145-163 as the same of synthetic peptide PII. From these results, Ab PII (145-163) was selected to probe for the further detection of PrP^C in each purification step of animal brain tissues.

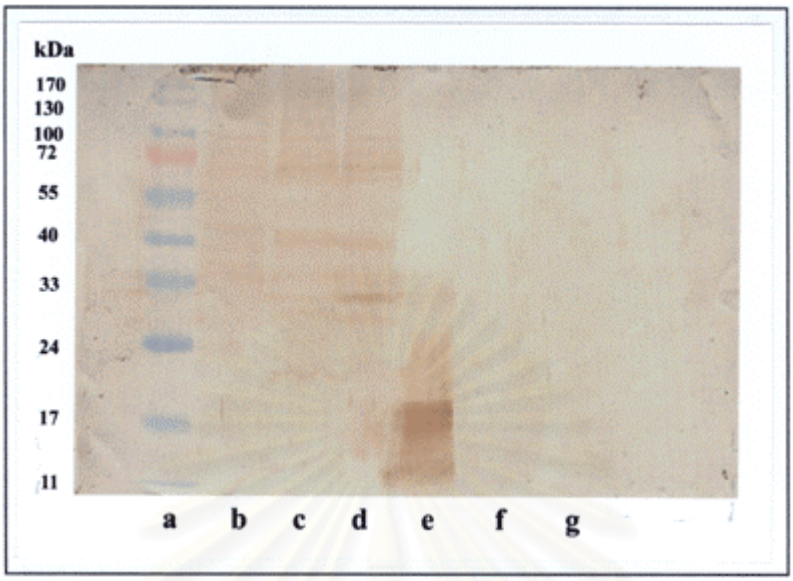

Figure 3.3(a) Immunodetection of cellular PrP in crude brain homogenates by mouse polyclonal antisera. Crude brain homogenates were separated by 12% gel SDS-PAGE, transferred to nitrocellulose membrane and immunostained with Ab PI (40-56).

Lane a = Prestained protein ladder

Lane b = Crude brain homogenates of fish, 66 μg

Lane c = Crude brain homogenates of pig, 52 μg

Lane d = Crude brain homogenates of cow, 68 μg

Lane e = Synthetic peptide I; PI (40-56), 20 μg

Lane f = Synthetic peptide II; PII (145-163), 20 μg

Lane g = Synthetic peptide III; PIII (209-228), 20 μg

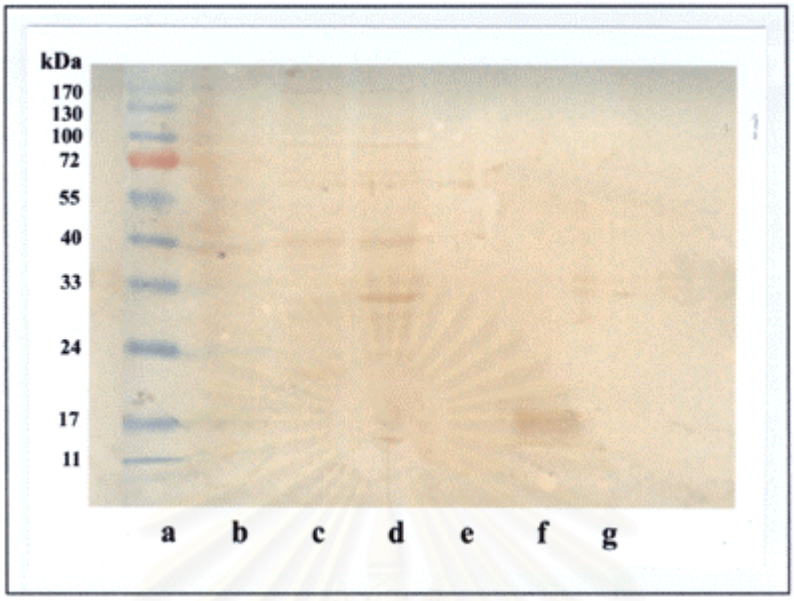

Figure 3.3(b) Immunodetection of cellular PrP in crude brain homogenates by mouse polyclonal antisera. Crude brain homogenates were separated by 12% gel SDS-PAGE, transferred to nitrocellulose membrane and immunostained with Ab PII (145-163).

Lane a = Prestained protein ladder

Lane b = Crude brain homogenates of fish, 66 μg

Lane c = Crude brain homogenates of pig, 52 μg

Lane d = Crude brain homogenates of cow, 68 μg

Lane e = Synthetic peptide I; PI (40-56), 20 μg

Lane f = Synthetic peptide II; PII (145-163), 20 μg

Lane g = Synthetic peptide III; PIII (209-228), 20 μg

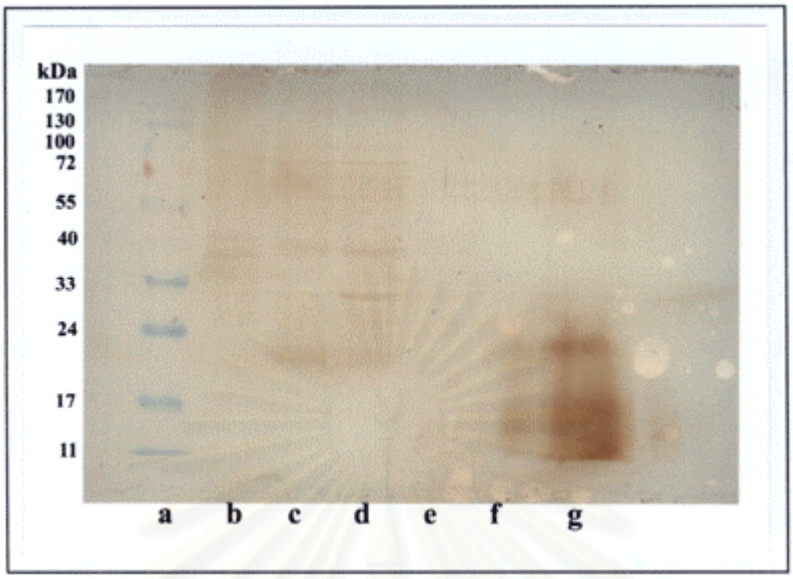

Figure 3.3(c) Immunodetection of cellular PrP in crude brain homogenates by mouse polyclonal antisera. Crude brain homogenates were separated by 12% gel SDS-PAGE, transferred to nitrocellulose membrane and immunostained with Ab PIII (209-228).

Lane a = Prestained protein ladder

Lane b = Crude brain homogenates of fish, 66 μg

Lane c = Crude brain homogenates of pig, 52 μg

Lane d =Crude brain homogenates of cow, 68 μg

Lane e = Synthetic peptide I; PI (40-56), 20 μg

Lane f = Synthetic peptide II; PII (145-163), 20 μg

Lane g = Synthetic peptide III; PIII (209-228), 20 μg

Figure 3.3(d) Immunodetection of cellular PrP in crude brain homogenates by monoclonal antibody; mAb L42. Crude brain homogenates were separated by 12% gel SDS-PAGE, transferred to nitrocellulose membrane and immunostained with mAb L42.

Lane a = Prestained protein ladder

Lane b = Crude brain homogenates of fish, 66 μg

Lane c = Crude brain homogenates of pig, 52 μg

Lane d = Crude brain homogenates of cow, 68 μg

Lane e = Synthetic peptide I; PI (40-56), 20 μg

Lane f = Synthetic peptide II; PII (145-163), 20 μg

Lane g = Synthetic peptide III; PIII (209-228), 20 μg

3.5 Immunoblot detection of cellular PrP in animal brain tissues

Each purification step in section 3.3 was analyzed by 12% gel of denaturing polyacralamide gel electrophoresis and stained for protein by Coomassie blue staining or immunostaining on nitrocellulose membrane according to the methods in section 2.10.5 or 2.12. The results are shown in ***Figure 3.4***, ***3.5***, and ***3.6.*** PrP^C from brain detergent extract of pig and cow, partially purified by immobilized metal affinity chromatography (IMAC) was found in either imidazole or EDTA eluate fraction of IMAC column. Two PII immunostained protein bands (30 kDa and 40 kDa in pig and cow; 40 kDa and 42 kDa in fish) from the 10 mM imidazole (Buffer I) eluate fraction (lane e) appeared at the corresponding molecular weights in the crude brain homogenates (lane b) and the supernatant of low-speed centrifugation (lane c) in ***Figure 3.4, 3.5, and 3.6***. In the EDTA eluate fraction of pig and cow brains (lane f), an approximately 21 kDa protein detected by Coomassie Blue staining was identified as PrP by PII immunostaining. A 21 kDa from the EDTA eluae fraction of fish brain, as detected by Coomassie Blue staining, however, was not stained by PII antiserum. The immunoblot assay of the supernatant after PEG precipitation step in all cases (***Figure 3.4, 3.5 and 3.6*** lane d) showed no immunostaining of the protein bands visualized by Coomassie Blue. The total protein in the EDTA eluate fraction normalized by the starting brain mass was approximately 7.6 μg/g, 8.7 μg/g and 2.2 μg/g for fish, pig and cow brain, respectively.

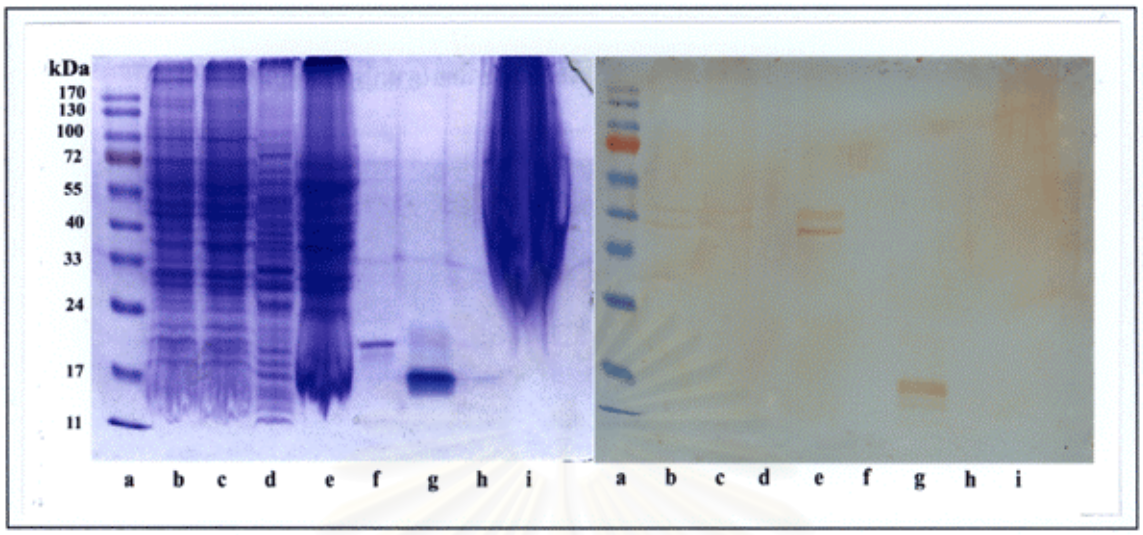

Figure 3.4 Preparation of detergent-solubilized microsomal fraction from fish brain homogenates. Protein samples were separated by 12% gel SDS-PAGE and analyzed by (left) Coomassie Blue staining or (right) transferred to nitrocellulose membrane and immunostaining with Ab PII (145-163).

Lane a = Prestained protein ladder

Lane b= Crude brain homogenate, 66 μg

Lane c = Supernatant of low-speed centrifugation, 58 μg

Lane d = Supernatant after PEG-8000 precipitation, 42 μg

Lane e = Eluate fraction by 10 mM imidazole in phosphate buffer, pH 7.0 (Buffer A), 40 μg

Lane f = Eluate fraction by 50 mM EDTA in phosphate buffer, pH 7.0 (Buffer D), 2.2 μg

Lane g = Synthetic peptide; P II, 20 μg

Lane i = Supernatant of ZW 3-12 solubilized fraction, 110 μg

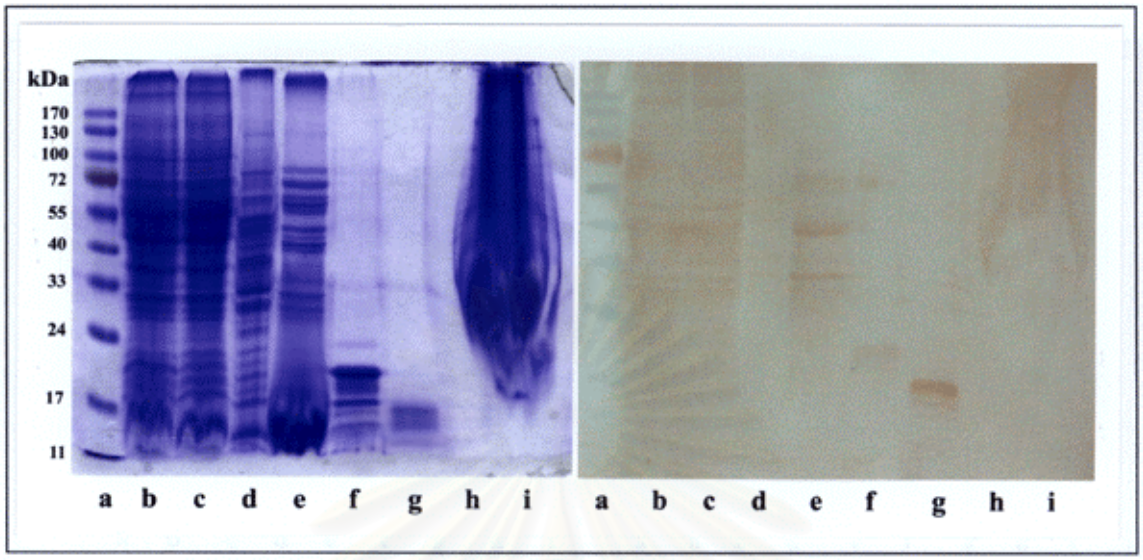

Figure 3.5 Preparation of detergent-solubilized microsomal fraction from pig brain homogenates. Protein samples were separated by 12% gel SDS-PAGE and analyzed by (left) Coomassie Blue staining or (right) transferred to nitrocellulose membrane and immunostaining with Ab PII (145-163).

Lane a = Prestained protein ladder

Lane b = Crude brain homogenate, 52 μg

Lane c = Supernatant of low-speed centrifugation, 48 μg

Lane d = Supernatant after PEG-8000 precipitation, 30 μg

Lane e = Eluate fraction by 10 mM imidazole in phosphate buffer, pH 7.0 (Buffer A), 40 μg

Lane f = Eluate fraction by 50 mM EDTA in phosphate buffer, pH 7.0 (Buffer D), 6 μg

Lane g = Synthetic peptide; P II, 20 μg

Lane i = Supernatant of ZW 3-12 solubilized fraction, 100 μg

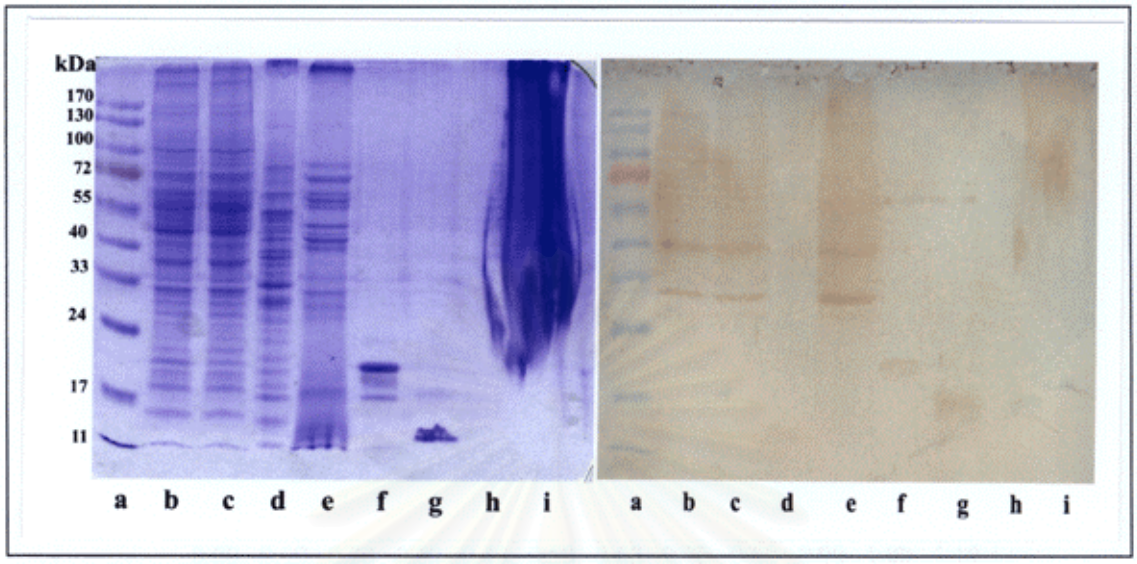

Figure 3.6 Preparation of detergent-solubilized microsomal fraction from cow brain homogenates. Protein samples were separated by 12% gel SDS-PAGE and analyzed by (left) Coomassie Blue staining or (right) transferred to nitrocellulose membrane and immunostaining with Ab PII (145-163).

Lane a = Prestained protein ladder

Lane b= Crude brain homogenate, 68 μg

Lane c = Supernatant of low-speed centrifugation, 50 μg

Lane d = Supernatant after PEG-8000 precipitation, 44 μg

Lane e = Eluate fraction by 10 mM imidazole in phosphate buffer, pH 7.0 (Buffer A), 50 μg

Lane f = Eluate fraction by 50 mM EDTA in phosphate buffer, pH 7.0 (Buffer D), 3 μg

Lane g = Synthetic peptide; P II, 20 μg

Lane i = Supernatant of ZW 3-12 solubilized fraction, 100 μg

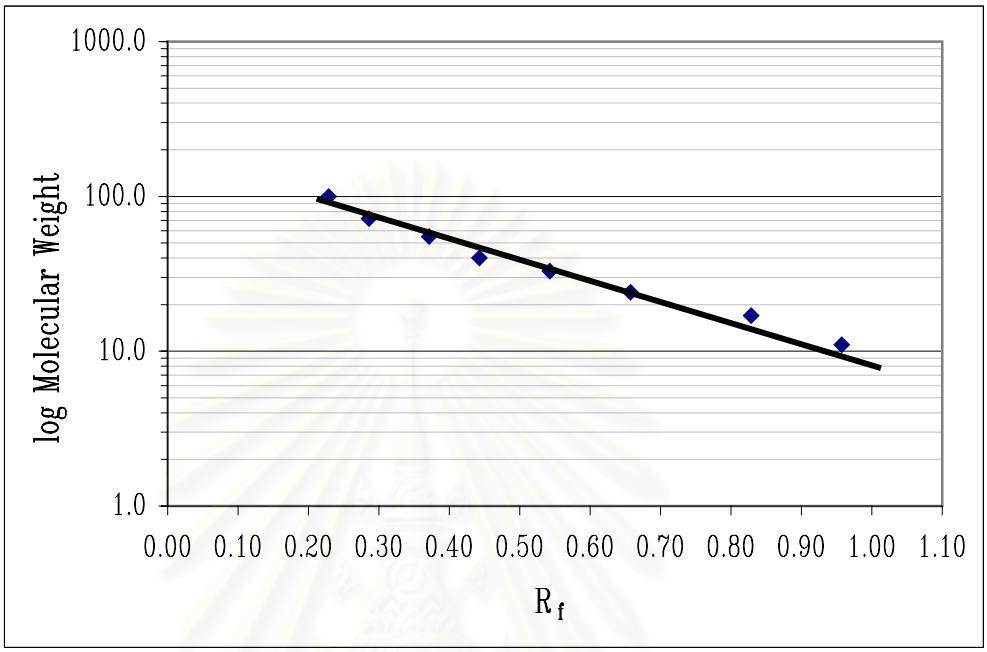

Figure 3.7 Calibration for molecular weight on 12% SDS-PAGE of Prestained Protein Ladder, ~10-180 kDa

CHAPTER IV
DISSCUSSION

4.1. Production of synthetic peptides and generation of mouse polyclonal antisera

In this study, the generation of polyclonal antisera, mice were immunized with synthetic peptides according to three different regions of amino acid sequences of sheep PrP. Three synthetic peptides, PI (a.a. residues 40-56), PII (a.a. residues 145-163), and PIII (a.a. residues 209-228) were selected to synthesize because the previous studies have revealed that polyclonal antisera were stimulated with three regions showed high antibody titers in ELISA and reacted with PrP^C in immunoblot (Harmeyer *et al.*, 1998). Furthermore, mAb directed against the amino acid sequence 145-163 exhibited a broad cross-reactivity with immunoblotted PrP^C of different mammalian species. Spectroscopic data of PrP structure have demonstrated that the synthetic peptide, PI (40-56) lies in the conserved region of flexibly disordered N-terminal segment of PrP^C. While the other two synthetic peptides, PII (145-163) and PIII (209-228) are the fragments in the region of C-terminal segment that form a globular domain where these two peptides constitute in the region of the first and last α-helix of 3-helices of PrP^C. These three synthetic peptides were custom synthesized by using an automated peptide synthesizer and multiple antigenic peptides (MAP) system as described in Section 2.6. This method has been reported to elicit a stronger immune response without the need for a carrier protein than the conventional technique using single monomeric peptides (Tam, 1988).

Using ELISA, the titers of mouse (BALB/cA) antibodies that had been raised against sheep prion fragment sequences were found in the ascending order PI<PII <PIII (***Table 4.1*** last row). The same ascending pattern was also reported in C57BL/6

mouse (Harmeyer *et al.*, 1998). This titer pattern could be explained by noting the decrease in sequence homology between sheep and mouse prions going from PI to PII to PIII in ***Figure 1.2***.

Raising antibodies against essentially the same prion fragment, the resulting titer for PI (1:1,600 in BALB/cA mouse) was significantly lower than reported figures in C57BL/6 mouse (1:25,600 to 1:51,200; Harmeyer *et al.*, 1998), rabbit (1:204,800; Groshup *et al.*, 1997), and chicken (1:6,400; Groshup *et al.*, 1997). In spite using of MAP system, this lower antibody titer of PI because the amino acid sequence in this region (40-56) of mouse (vaccinated species) and sheep (donor species) PrP seems to be conserved. For the titer of multiple antigenic peptide; PIII (1:1,638,400 in BALB/cA mouse) showed the highest comparing to resulted that were previously published obtained with monomeric peptide reported by Harmeyer *et al.* (1998) and Groschup *et al.* (1997) as shown in ***Table 4.1***.

4.2 Extraction and partial purification of cellular prion protein (PrP^C) from animal brain tissues

In this study, the ligand exchanges and chelate annihilation phenomenon already described in Section 1.6 (Sulkowski, 1985) was observed since imidazole buffer released a fraction of prions from the IMAC column (***Figure 3.4, 3.5, and 3.6*** lane e, right) along with several other non-prion proteins that was detected by Coomassie Blue under SDS-PAGE (***Figure 3.4, 3.5, and 3.6*** lane e, left).

In EDTA eluate fraction the amount of PrP^C is approximately 8.7 μg/g pig brain tissues and 2.2 μg/g cow brain tissues. According to the literature Syrian hamster (SHa) PrP^C is expressed at levels of approximately 70 μg/g brain tissue or

Table 4.1 Comparison of Antibody titers (OD_{50}) between MAP system and monomeric peptide from previous reports

Donor species	Vaccinated species	System	Antibody titers PI (residues)	PII (residues)	PIII (residues)	References
mouse	rabbit	MAP	——	——	**1:1,024,000** (213-226)	Yokoyama *et al.*, 1995
sheep	rabbit	monomeric	**1:204,800**	**1:102,400**	**1:204,800**	Groshup *et al.*,
	chicken	monomeric	**1:6,400** (40-56)	**1:12,800** (145-163)	**1:2,260** (209-228)	1997
sheep	mouse1	monomeric	**1:51,200**	**1:102,400**	**1:102,400**	Harmeyer *et al.*,
	mouse2 (C57BL/6)		**1:25,600** (40-56)	**1:18,080** (145-163)	**1:102,400** (209-228)	1998
sheep	mouse (BALB/cA)	MAP	**1:1,600** (40-56)	**1:51,200** (145-163)	**1:1,638,400** (209-228)	From this study (***Table 3.2***)

0.01% of the proteins in SHa brain indicated that PrP^C is a rare protein in the brain (Groschup *et al.*, 1997; Harmeyer *et al.*, 1998).

4.3 Detection of cellular prion protein (PrP^C) from various animal species

For the detection of PrP^C from various animal species, antibody directed aginst PII (145-163) was selected for detecting the PrP^C in each purification step because the antisera against this peptide fragment was previously reported to cross-react with PrP^C of various mammalian species (Harmeyer *et al.*, 1998). From this result, Ab PII (145-163) cross-reacted with a protein band of molecular mass (M_r) approximately 40 kDa in crude brain homogenates of fish, pig and cow elucidated in immunoblot assay (***Figure 3.4, 3.5, and 3.6*** lane b) and especially in crude brain homogenates and the low-speed supernatant of pig and cow (***Figure 3.5, and 3.6*** lane c) where the other protein band at M_r about 30 kDa was found. In addition, the EDTA eluate fraction from IMAC column contained exclusively the identical band of approximately M_r 21 kDa in the pig and the cow (***Figure 3.5, and 3.6*** lane f). Although in crude brain homogenate of fish it was found to certain a broad band at M_r 21 kDa in Coomassie blue staining but was not detected in immunostaining (***Figure 3.4*** lane f) and even it was confirmed with another polyclonal antisera; Ab PI (40-56) and Ab PIII (209-228) (data not shown). However, PrP that was not detected in the EDTA eluate fraction do not yet confirm that fish could be able to have the prion protein. Since Gibbs and Bolis (1997) reported that they first detected the normal prion protein in the brains of spawning salmon fish collected in Alert Bay, Canada. Recent research by Milla *et al*. (2003) revealed a novel prion gene in Japanese pufferfish *Fugu rubripes* where a gene that encodes a structurally conserved prion protein was cloned.

There is evidence supporting the existence of three full-length PrP^C glycoforms; the unglycosylated form, which migrates at 27 kDa, the intermediate form, thought to be monoglycosylated, which migrates at 28-30 kDa, and the highly glycosylated form, which migrates as a band spanning 33-42 kDa. In addition, PrP^C which are truncated at the N-terminus and generated during normal processing of PrP^C migrates as 25 kDa in glycosylated form and as 18 kDa in unglycosylated form (Zanusso *et al.*, 1998). From the obtained data (***Figure 3.5 and 3.6***), the protein bands at M_r 40 kDa and 30 kDa in crude brain homogenates and low-speed supernatant of pig and cow might represent the highly or di-glycosylated form and monoglycosylated form of PrP^C, respectively by glycosylation at the one or both sites of Asn-181 and Asn-197. As shown in ***Figure 4.1***, these two proteins appearing in the imidazole eluate fraction (***Figure 3.5 and 3.6*** lane e) should be derived by proteolytic cleavage at a site near or beyond the octapeptide repeats region which ends approximately at residue 90 of PrP. Removal of this copper-binding region results in releasing PrP^C from the IMAC column in the presence of imidazole. In the EDTA eluate fraction (***Figure 3.5 and 3.6*** lane f), the protein band at M_r 21 kDa in pig and cow might represent the PrP that has been cleaved near amino acid residue163 because Ab PII (145-163) was able to detect this protein in immunoblot.

From several evidence, the peptide antisera is a valuable tools for the immunochemical characterization cellular isoform (PrP^C) as well as pathological isoform (PrP^{Sc}), however to date, the synthetic PrP peptide antisera fail to discriminate between PrP^C and PrP^{Sc} (Harmeyer *et al.*, 1998), suggesting that the chemical characteristics which differentiate these two PrP isoform are not associated which the polypeptide epitopes identified by these antisera (Barry *et al.*, 1988). Nevertheless, these antibodies could serve as sensitive and highly specific probes in

diagnosis investigations as well as in studies on the pathogenesis of TSEs in man and other animals.

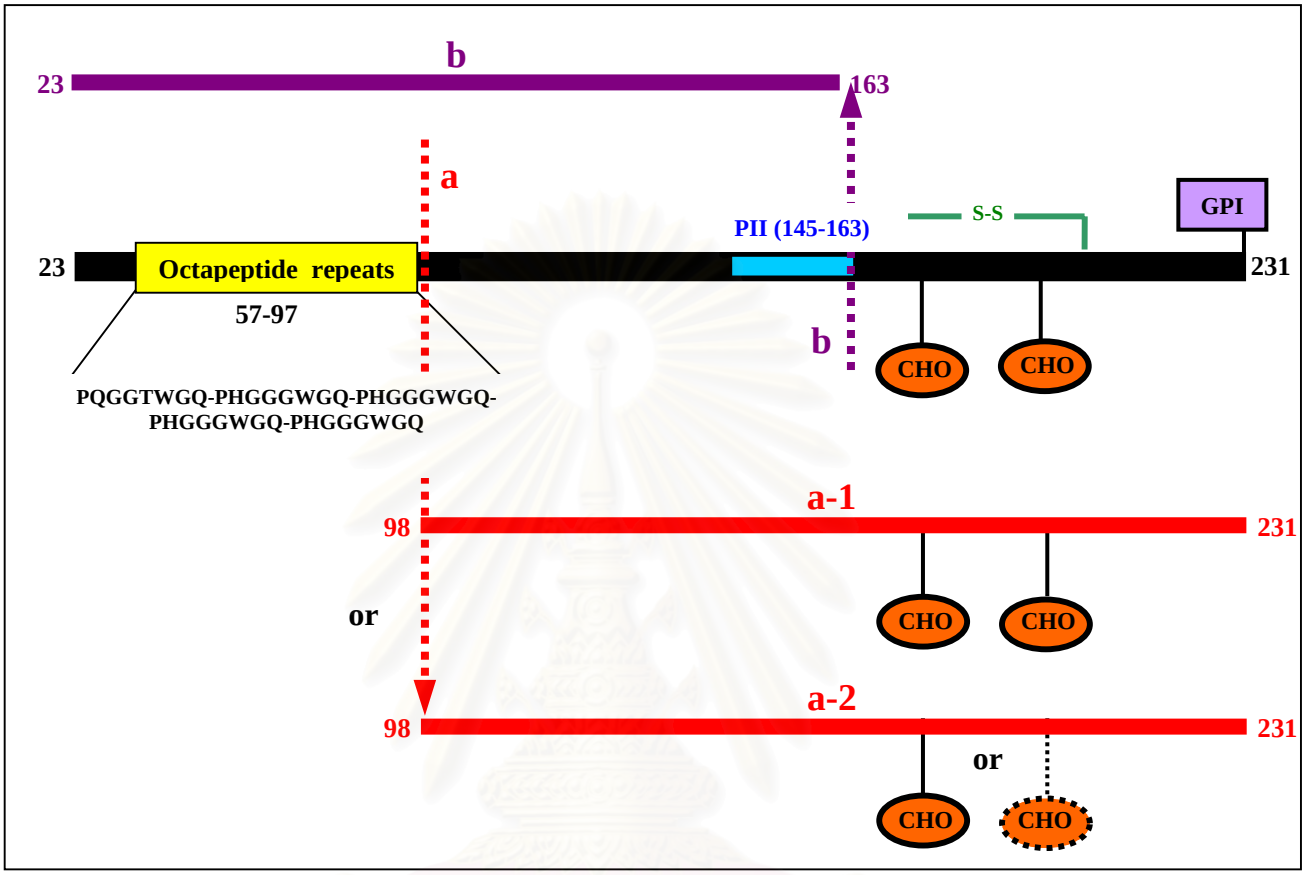

Figure 4.1 Proposed structures of proteolytic cleavage of prion protein.

The 40 kDa and 30 kDa bands fron pig and cow brains, which were stained by Ab PII, might have resulted from a single proteolytic cleavage at site a, which is near or beyondthe end of the octapeptide repeats (residue 57-97). These fragments were eluted with the imidazole buffer since they lack the octapeptide repeats that bind Cu^{2+} ions on the column. The 40 kDa peptide is shown diagrammatically to be more glycosylated than 30 kDa peptide. The 21 kDa peptide might have resulted from a single proteolytic cleavage at site b, which is somewhere between the end of PII (residue 145-163) and a disulfide linkage (Cys 179). This fragment contains the entire octapeptide repeats and was therefore eluted from the column with EDTA.

CHAPTER V
CONCLUSION

1. Mouse polyclonal antisera generated in laboratory for this study were raised in BALB/cA mice against three MAPs corresponding to sheep PrP amino acids sequence; PI (40-56), PII (145-163), and PIII (209-228).

2. The antisera elicited by each peptide displayed antibody binding to the homologous peptide under Indirect Enzyme-linked Immunosorbent Assay (indirect ELISA). Antisera against sequence PI, PII, and PIII showed antibody titers of 1:1,600, 1: 51,200 and 1:1,638,400, respectively.

3. Antiserum against multiple antigenic peptide (MAP); PIII showed an order of magnitude higher antibody titers comparing previously published results that were obtained with monomeric peptide conjugated with carrier proteins. Antiserum against sequence PI gave low antibody titers. This was probably due to the evolutionary conserved sequence of PI.

4. PrP^{C} was purified from microsomal fraction by detergent extraction, differential centrifugation, and immobilized Cu^{2+} ion affinity chromatography (IMAC). The form eluted by 10 nM imidazole might be the diglycosylated and monoglycosylated PrP while that eluted by EDTA might be the PrP truncated C-terminus.

5. PrP^{C} could be detected in crude brain homogenates with Ab PI, Ab PII, and Ab PIII. In crude brain homogenates and low-speed supernatant of the pig, and the cow were found the protein band appeared at the molecular mass (M_r) 40 kDa and

the other protein band of approximate M_r 30 kDa which was not observe in crude brain homogenates of fish in immunoblot.

6. In EDTA eluate fraction showed a protein band at 21 kDa of the pig and the cow in both Coomassie blue staining and immunostaining but not in fish even it was confirmed with another polyclonal antisera; Ab PI and Ab PIII.

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APPENDICES

Appendix 1: Preparation for polyarylamide gel electrophoresis

1. Stock reagents

Solution A (30% Acrylamide, 0.8% Bis-acrylamide), 100 ml

Acrylamide	29.2 g
N,*N*'-methylene-bis-acrylamide	0.8 g

Adjusted volme to 100 ml with distilled water

Solution B

2 M Tris-HCl, pH 8.8	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C

1 M Tris-HCl, pH 8.8	50 ml
10% SDS	4 ml
Distilled water	46 ml

2 M Tris-HCl, pH 8.8

Tris(hydroxymethlene)-aminomethylene 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1.5 M Tris-HCl, pH 8.8

Tris(hydroxymethlene)-aminomethylene 18.17 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1M Tris-HCl, pH 6.8

Tris(hydroxymethlene)-aminomethylene 12.1 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

0.5 M Tris-HCl, pH 6.8

Tris(hydroxymethlene)-aminomethylene 6.06 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

2. SDS-PAGE

15% separating gel

Solution A	3.75 ml
Solution B	2.5 ml
Distilled water	1.14 ml
10% $(NH_4)_2S_2O_8$	50 μl
TEMED	10 μl

5% stacking gel

Solution A	0.84 ml
Solution C	1 ml
Distilled water	3.1 ml
10% $(NH_4)_2S_2O_8$	50 μl
TEMED	10 μl

Sample buffer

1 M Tris-HCl,pH 6.8	0.6 ml
50% glycerol	5.0 ml
10% SDS	2.0 ml

2-mercaptoethanol	0.5 ml
1% bromphenol blue	1.0 ml
Distilled water	0.9 ml

One part of sample buffer wad added to four part of sample. The mixture was heated 5 minutes in boiling water before loading to the gel.

Electrophoresis buffer, 1 liter

Tris(hydroxymethyl)-aminomethane	30 g
Glycine	144 g
SDS	10 g

Adjusted volume to 1 liter with distilled water (pH should be approximately 8.3)

Coomassie blue stain solution, 1 liter

Coomassie blue R-250	1.0 g
Methanol	450 ml
Glacial acetic acid	10 ml
Distill water	450 ml

Stirred Coomassie blue and methanol for about 3 h, after that added acetic acid and distilled water, mixed well and then filtered this solution.

Destaining solution, 3 liters

Methanol	300 ml
Glacial acetic acid	300 ml
Distilled water	2400 ml

Appendix 2: Preparation of buffer for immunoblotting assay

Tank-blotting transfer buffer, 1 liter

25 mM Tris(hydroxymethyl)-aminomethane	3.03 g
150 mM Glycine	11.26 g
20% Methanol	200 ml

Adjusted volume to 1 liter with distilled water (pH should be approximately 8.3)

TBS buffer, 500 ml

0.2 M Tris-HCl, pH 7.5	25 ml
1 M NaCl	75 ml
Distilled water	400 ml

TBS-Tween buffer, 500 ml

0.2 M Tris-HCl, pH 7.5	50 ml
1 M NaCl	250 ml
Tween 20	0.125 μl
Distilled water	200 ml

Blocking buffer, 100 ml

BSA (3% w/v)	3 g
TBS buffer	100 ml

10x Tris-saline, 100 ml

NaCl (9% w/v)	9 g
1 M Tris-HCl, pH 8.8	100 ml

Staining solution

3,3'-Diaminobenzidine	50 mg
30% Hydrogen peroxide	60 µl
1x Tris-HCl, pH 8.0	30 ml

0.2 M Tris-HCl, pH 7.5

Tris(hydroxymethlene)-aminomethylene 12.114 g

Adjusted pH to 7.5 with 1 M HCl and adjusted volume to 500 ml with distilled water.

1 M Tris-HCl, pH 8.0

Tris(hydroxymethlene)-aminomethylene 36.34 g

Adjusted pH to 8.0 with 1 M HCl and adjusted volume to 300 ml with distilled water.

Appendix 3: Preparation of buffer for Enzyme-linked immuno-sorbent assay (ELISA)

Blocking buffer

BSA (3% w/v)	3 g
PBS-Tween	100 ml

Washing buffer

Phosphate buffer saline (PBS), pH 7.4	100 ml
Tween 20 (0.1%)	0.1 ml

Substrate solution

3,3',5,5'-Tetramethlbenzidine (TMB)	1.0 mg
Dimethysulfoxide (DMSO)	100 μl
0.1 M Sodium acetate/citric buffer, pH 5.0	9.9 ml
Hydrogen peroxide	0.1 mg

1.0 mg of TMB was first dissolved in DMSO, then dilute with 0.1 M sodium acetate/citric buffer, pH 5.0. It was mixed gently and wait for 10 min before adding to the hydrogen peroxide. This substrate solution must be prepares freshly before using.

Stop solution

2 M Sulfuric acid, 100 ml

Sulfuric acid	10.8 ml
Distilled water	89.2 ml

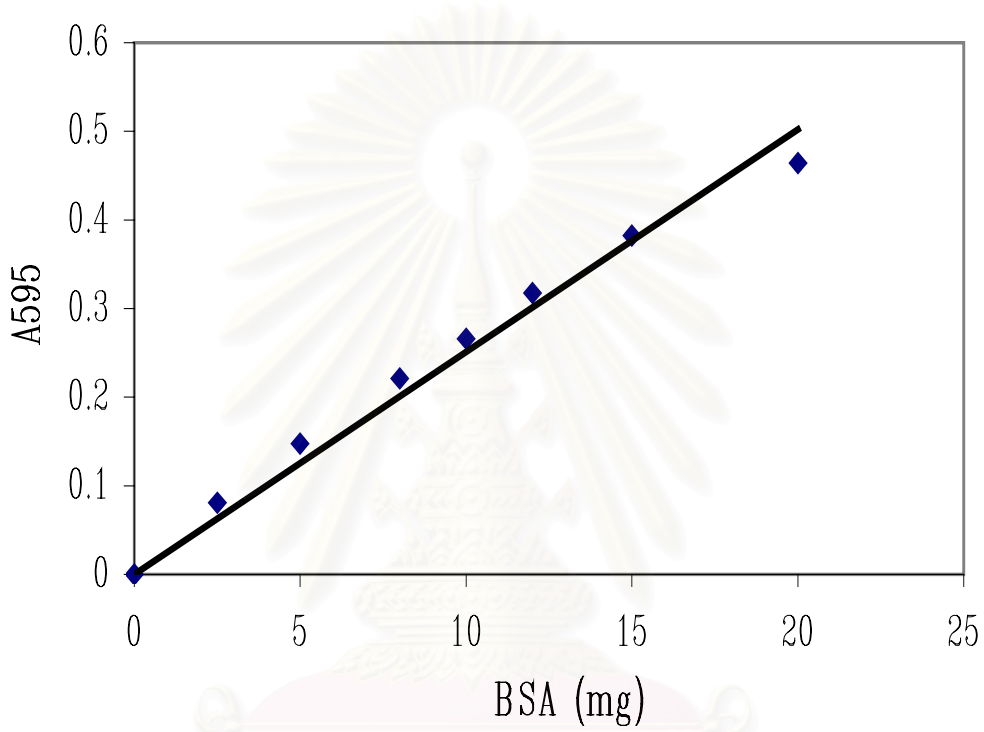
Add sulfuric acid into distilled water.

4x 0.1 M Sodium acetate/citric buffer, pH 5.0

Citric acid	1.05 g
Sodium acetate	1.64 g

Add distilled water to make volume 250 ml

Appendix 4: Standard curve for determination by Bradford's method



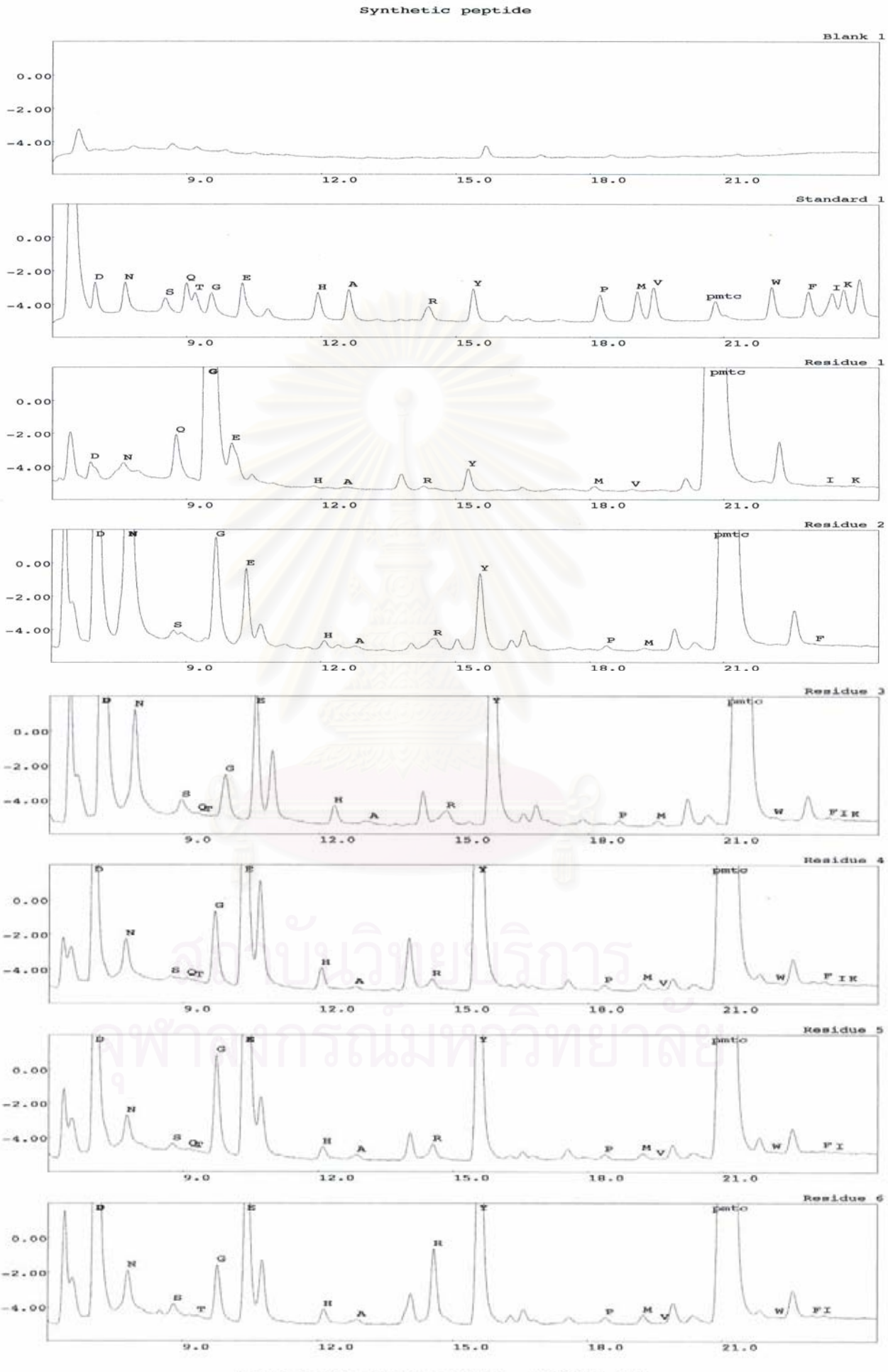
Appendix 5: Abbreviation for amino acid residues

Amino acid	3 Letter-Abbreviation	1 Letter-Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Valine	Val	V

Appendix 6: N-terminal Peptide sequencing

AAacid #	AAacid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-back)	Pmol (+lag)	AAcid ID
1	G	9.48	9.55	48.53	46.88	50.01	Gly
2	N	7.70	7.62	15.59	13.84	15.65	Asn
3	D	7.16	6.95	20.85	19.38	23.32	Asp
4	Y	15.54	15.39	20.03	10.13	10.13	Tyr
5	E	10.38	10.23	15.02	11.91	13.87	Glu
6	D	7.06	6.95	15.22	13.94	16.56	Asp
7	R	14.71	14.39	13.34	12.66	15.23	Arg
8	Y	15.39	15.39	14.07	6.62	7.72	Tyr
9	E	10.46	10.23	3.27	1.02	1.23	Glu
10	R	14.14	14.39	10.17	9.58	11.44	Arg
11	E	10.29	10.23	5.18	3.35	3.97	Glu
12	N	7.69	7.62	5.87	4.14	4.95	Asn
13	M	19.12	19.05	2.09	1.87	2.25	Met
14	Y	15.40	15.39	5.03	1.27	1.53	Tyr
15	R	14.43	14.39	3.59	3.14	3.77	Arg
16	Y	15.56	15.39	3.80	1.26	1.28	Tyr
17	P	18.28	18.22	3.28	1.32	1.54	Pro
18	N	7.83	7.62	2.15	0.44	0.52	Asn
19	Q	9.20	8.99	1.27	0.63	0.73	Gln
20	Q	8.97	8.99	1.59	0.92	0.81	Gln

Sequence: **GNDYEDRYERENMYRYPNQQ**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Standard 1
Residue 1
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Residue 3
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9.0
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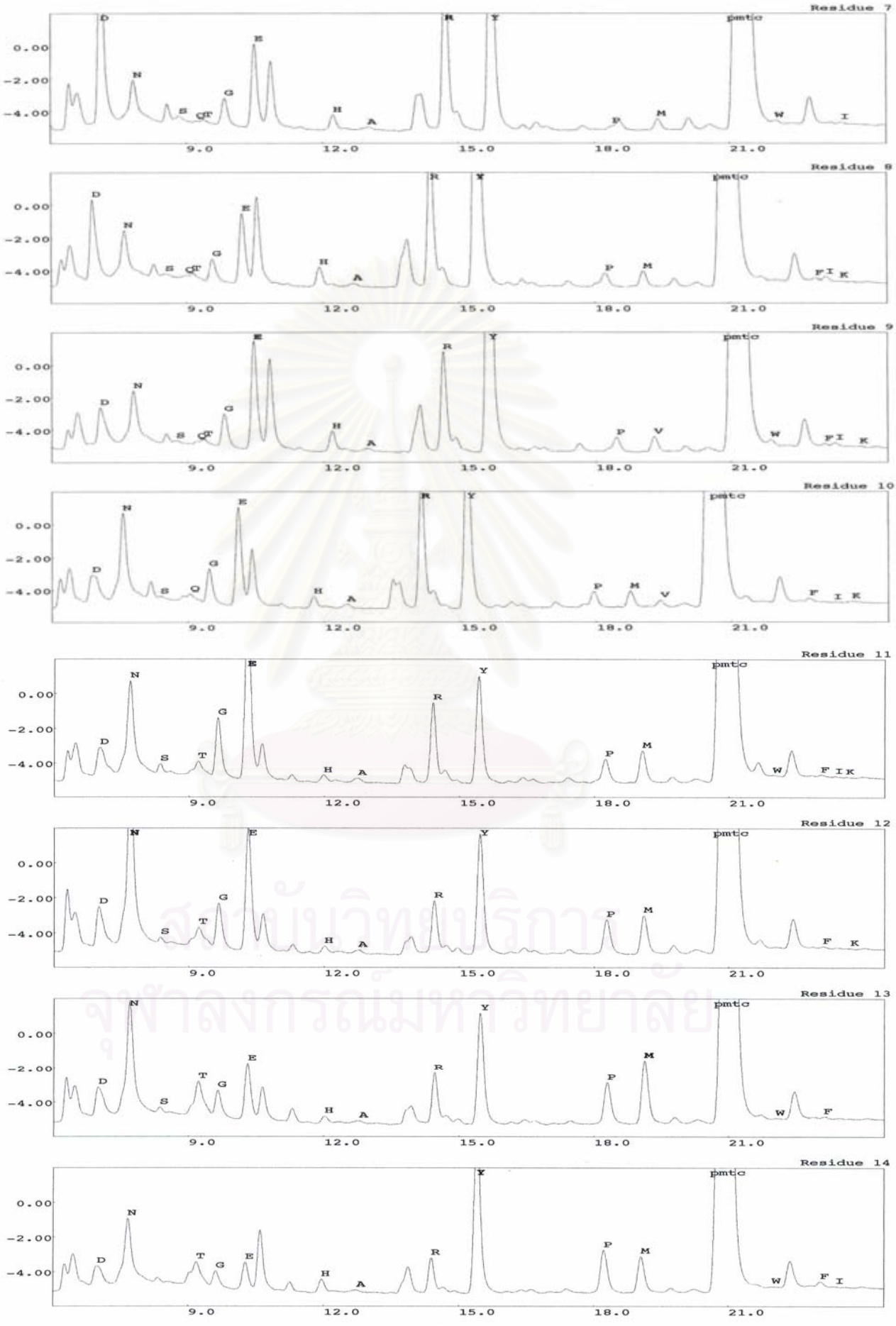
15.0
18.0
21.0
Applied Biosystems Procise - PROCISE-cLC

7/4/04 10:19 Synthetic peptide



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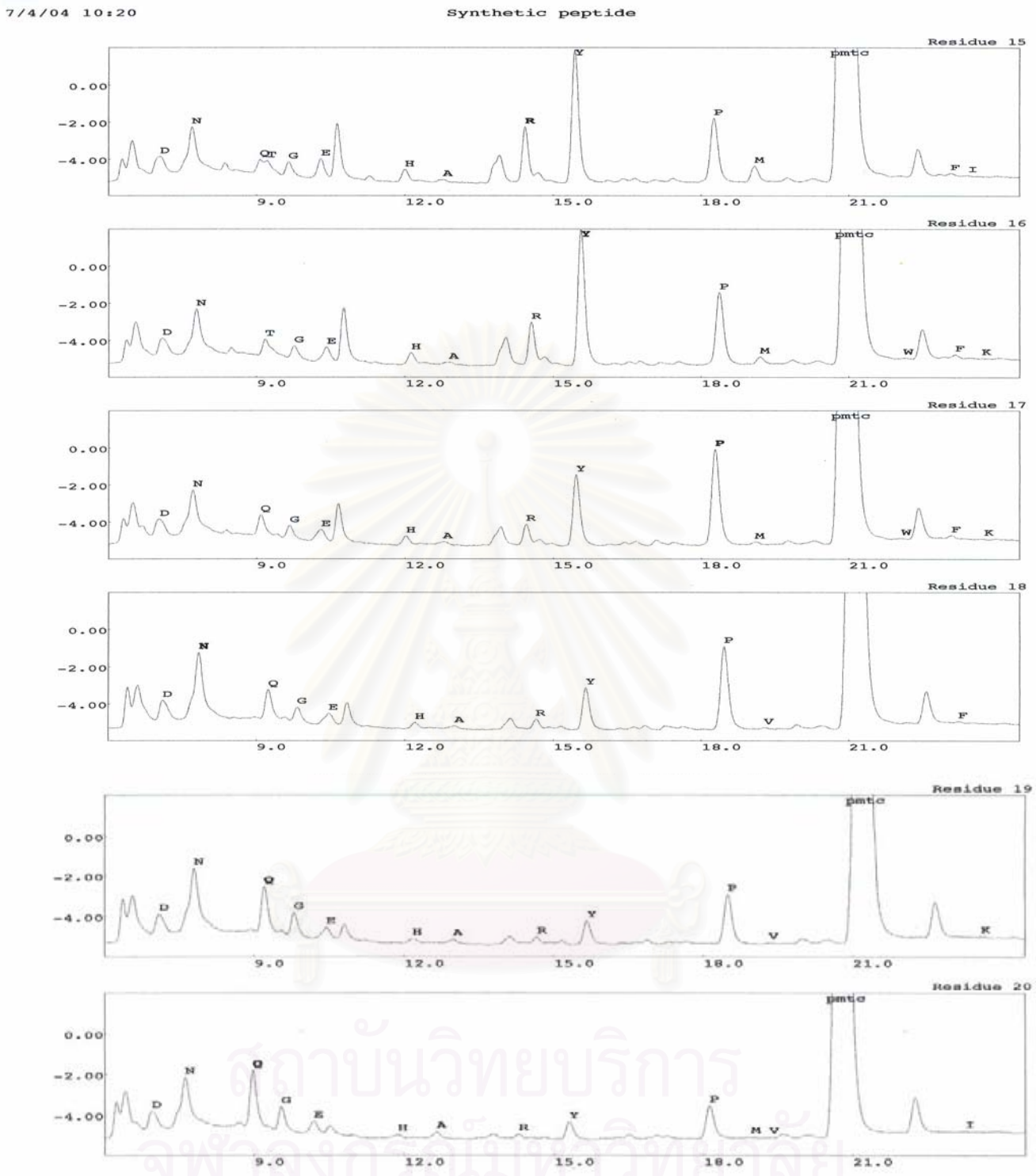

Appendix 7: Preparation of Bradford solution

Coomassie blue G-250	100	mg
Ethanol	50	ml
85% Phosphoric acid	100	ml

Dissolved Coomassie blue and ethanol for about 2-3 hours after that adding 85% phosphoric acid. Adjusted volume to 1 liter with distilled water and filtered this solution.

BIOGRAPHY

Miss Srikul Kantajai was born on June 5, 1976 in Bangkok. She graduated with the Bachelor Degree of Science in Chemistry from Ramkhamhang University in 1999. She continued studying for Master Degree in Biochemistry Program at Chulalongkorn University.