กิจกรรมการยับยั้งเอนไซม์แอลฟา-อะไมเลสของสารสกัดถั่วขาว

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ALPHA-AMYLASE INHIBITION ACTIVITY OF WHITE KIDNEY BEAN (*PHASEOLUS VULGARIS*) EXTRACT

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ALPHA-AMYLASE INHIBITION ACTIVITY OF WHITE KIDNEY BEAN (*Phaseolus vulgaris*) EXTRACT

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ถั่วขาวประกอบด้วยสารยับยั้งแอลฟา-อะไมเลสที่มีโปรตีนเป็นส่วนประกอบ ซึ่งรบกวนการย่อยคาร์โบไฮเดรตเชิงซ้อน กลุ่มเอนไซม์-สารยับยั้งสามารถลดพลังงานที่ได้รับจากคาร์โบไฮเดรต ลดการหลั่งอินซูลินและยับยั้งการหลั่งน้ำย่อยโพลีเปปไทด์ของกระเพาะอาหาร วิทยานิพนธ์นี้นำเสนอการศึกษาและออกแบบวิธีการที่เหมาะสมในการสกัดสารยับยั้งเอนไซม์แอลฟา-อะไมเลสจากถั่วขาว ผลการทดลองแสดงค่าโปรตีนสูงสุดที่ 6.49±0.09 มิลลิกรัมต่อกรัมของถั่วขาวและมีค่าเฉพาะกิจกรรมยับยั้งเอนไซม์ แอลฟา-อะไมเลสที่ 34.69±0.56 ต่อมิลลิกรัมของโปรตีนในสารสกัดหยาบ ปริมาณผลผลิตของสารสกัดถั่วขาวสูงที่สุดคือ 23.32 เปอร์เซ็นต์ซึ่งได้จากสภาวะการสกัดที่ความเข้มข้น 0.15 โมลาร์ของสารละลายฟอตเฟตบัฟเฟอร์ ในการสกัด 2 ชั่วโมง และการแยกตัว 60 นาที อย่างไรก็ตามค่าเฉพาะเจาะจงในการยับยั้งกิจกรรมของเอนไซม์แอลฟา-อะไมเลสสูงที่สุดเกิดขึ้นโดยสภาวะการสกัดที่ความเข้มข้น 0.08 โมลาร์ของสารละลายฟอตเฟตบัฟเฟอร์ ในการสกัด 1.5 ชั่วโมงและการแยกตัว 30 นาที

สารสกัดถั่วขาวที่ได้จากสภาวะที่เหมาะสม มีค่าเฉพาะเจาะจงในการยับยั้งกิจกรรมของเอนไซม์แอลฟา-อะไมเลสสูงที่สุด เมื่อเปรียบเทียบกับสารสกัดทางการค้าหลังจากผ่านระบบการย่อยพบว่าสารสกัดถั่วขาวที่ได้และสารสกัดทางการค้ามีค่าเฉพาะเจาะจงในการยับยั้งกิจกรรมของเอนไซม์แอลฟา-อะไมเลสลดลง นอกจากนี้รูปแบบของโพลีเปปไทของสารสกัดถั่วขาวทั้งสองใกล้เคียงกัน

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

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JUTARAT MANATWIYANGNKOOL : ALPHA - AMYLASE INHIBITION ACTIVITY OF WHITE KIDNEY BEAN (*Phaseolus vulgaris*) EXTRACT. THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 88 PP.

ALPHA - AMYLASE INHIBITION ACTIVITY OF WHITE KIDNEY BEAN (*Phaseolus vulgaris*) EXTRACT

White kidney beans (*Phaseolus vulgaris*) contain proteinaceous inhibitors of α-amylase which can interfere with complex carbohydrate digestion. The enzyme-inhibitor complex would reduce energy obtained from carbohydrate, reduce insulin, and reduce gastric inhibitory polypeptide secretion. This study aimed to discover a suitable extraction method for α-amylase inhibition from white kidney bean. The results showed that the highest protein content was 6.49±0.09 mg/g of beans and α-amylase specific inhibition activity was 34.69±0.56 per mg of protein of the crude extract. The highest yield of the extract was 23.32% obtained from the extraction condition at 0.15 M Phosphate buffer saline, extraction time for 2 hr, and separation 60 min. Moreover, The highest α-amylase specific inhibition activity of the extract was optimized at 0.08 M Phosphate buffer saline, 1.5 hr extraction, and 30 min separation.

The white kidney bean extract from the optimized condition had the best α-amylase specific inhibition activity when compared with the commercial white kidney bean extract. After *in vitro* digestion, the α-amylase specific inhibition activity of the

white kidney bean extract and the commercial white kidney bean extract was decreased. In addition, the polypeptide pattern of both extracts was similar.

School of Food Technology Student's Signature _____

Academic Year 2014 Advisor's Signature _____

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Jutarat Manatwiyangkool

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SYMBOLS AND ABBREVIATIONS

°C	=	degree of celcius
AI	=	amylase inhibitor
ANOVA	=	analysis of variance
AU	=	∝-amylase activity unit
AIU	=	∝-amylase inhibitor activity
BSA	=	Bovine serum albumin
$CaCl_2$	=	calcium chloride
DNS	=	dinitrosalicylic acid reagent
Fig	=	Figure
g	=	Gram
h	=	Hour
KCl	=	potassium chloride
kD	=	Kilodalton
Kg	=	Kilogram
Kh/ha	=	kilogram per hectare
M	=	Molar
mg	=	Milligram
$MgSO_4$	=	magnesium sulfate
Mr	=	molecular weight
μg	=	Microgram
min	=	minute
mL	=	Milliliter

SYMBOLS AND ABBREVIATIONS (Continued)

μL	=	Microliter
mmol	=	Millimole
NaCl	=	Sodium chloride
NaOH	=	Sodium hydrxide
pH	=	Potential of Hydrogen ion
PPA	=	Porcine pancreatic ∝-amylase
RSM	=	Respone surface method
UV	=	Ultraviolet

CHAPTER I

INTRODUCTION

1.1 Introduction

The potential dangers of obesity and its high prevalence in developed countries are well known: it has been indicated as a significant risk factor in a wide range of morbidities from apnea, dyslipidemia, hypertension and diabetes mellitus to coronary artery disease. The varying remedies currently available to control excess body weight include pharmacological preparations and dietary supplements intended to restrict energy absorbance and promote weight loss, many of them based on plant products. Particular attention has focused on the so-called "starch-blockers", notwithstanding the absence of reliable scientific evidence for their efficacy. These supplements are based largely on the protein concentrates of *Phaseolus vulgaris* or kidney bean, which are known to contain high levels of an α-amylase inhibitor also known as phaseolamin (α-AI). Theoretically, through the inhibition of α-amylase, they may promote weight loss by interfering with the breakdown of complex carbohydrates, thereby reducing, or at least slowing the digestion of starch by allowing them to pass undigested into the lower gastrointestinal tract. (Boniglia et al, 2008)

White kidney beans (*Phaseolus vulgaris*) is a member of the Leguminosae, tribe Phaseoleae, subfamily Papilionoideae. Cultivated forms are herbaceous annuals, which are determinate or indeterminate in growth habit. *Phaseolus vulgaris* L. was

originally a crop of the New World, but is now grown extensively in all major continental areas. Its production spans from 52°N to 32°S latitude. It is a major source of dietary protein throughout both Latin America and Eastern Africa, but per capita consumption is declining as population increases outdistance production. The conditions under which this annual, predominantly self-pollinated legume is grown are extremely variable. While production tends to be centered on smaller holdings, the cropping system used can vary from the highly-mechanized, irrigated, and intensive production of monoculture bush beans, to complex associations of indeterminate or climbing beans with corn, other cereals, sugar cane, coffee or plantain. Technical inputs in such multiple-cropping systems are often limited, with the result that crop yields can range from less than 500 kg ha^{-1} in parts of Latin America and Africa to as much as 5000 kg ha^{-1} under experimental conditions. The diversity of conditions under which beans are grown, coupled with highly-specific local preferences for particular seed types or colors have complicated attempts at bean improvement. As a result, greatest progress has been in breeding for the resolution of disease, insect and nutritional constraints, with only limited. The present review considers the origins of *P. vulgaris*, the cropping systems used for bean production, the agronomic and biological factors which most limit crop yield, and the traits usually considered in bean improvement programs. It will also consider the impact of recent molecular advances to crop improvement on the prospects for the improved production of this important grain legume (Graham and Ranalli, 1997). Archeological investigations showed that *Phaseolus vulgaris* originated on the American Continent, specifically in southern United States, Mexico, Central America, and the northern part of South America. In particular, the species *P. vulgaris* was introduced into Europe in the sixteenth century and since then it has become a very important crop in many regions of the world (Carai et al, 2009).

Recently, common bean is gaining increasing attention as a functional or nutraceutical food, due to its rich variety of phytochemicals with potential health benefits such as fiber, polyphenolic compounds, lectins, unsaturated fatty acids, trypsin inhibitors, phytic acid, among others. Important biological activities have been described for fiber, polyphenolic compounds, lectins, trypsin inhibitors, and phytic acid from common beans like enhancement of the bifidogenic effect; antioxidant; antimutagenic; anticarcinogenic effects; as well as an antiproliferative effect on transformed cells. (Reynoso-Camacho, R , Ramos-Gomez, M and Loarca-Pina, G., 2006)

More attention is now being payed to these phaseolamins for two main reasons. First, they widely occur in both human and cattle diets and could therefore diminish the digestibility of starch and starch-derived products by inhibiting the α-amylase enzymes (Le berre-Anton et al, 1997). The use, in the early 1980s, of crude extracts of kidney bean as starch blockers to control human non-insulin-dependent diabetes mellitus and obesity, was hampered by their very low inhibitor content and the presence of potentially harmful lectins (PHA) and trypsin inhibitors (Leiner et al, 1984). Nevertheless, further investigations on humans showed that purified α-AI perfused into the duodenum significantly inhibited intraluminal amylase activity while ingested with dietary starch, it significantly reduced the postprandial increase in glucose of both normal and diabetic patients (Layer et al. 1985). Second, their use as insecticidal proteins to prevent the attack of predatory insects to susceptible seeds. (Ishimoto et al, 1995) Because α-AI inhibits the development of bruchid beetles, its gene is considered as potentially useful for crop protection via plant genetic engineering.

The presence of α-amylase inhibitors in beans (*Phaseolus vulgaris*), a common source of protein throughout the world, was first reported by Bowman (1945). α-amylase inhibitor (αAI) is present in embryonic axes and cotyledons, but not in other organs of the plant. (Moreno et al., 1990; Obiro et al., 2008) In *Phaseolus vulgaris* contains an α-amylase inhibitor is a glycoprotein (about 15% carbohydrate) that inhibits the activity of mammalian and insect α-amylases, but not of plant α-amylases. The protein is synthesized during the same time period that phaseolin and phytohemagglutinin are made and also accumulates in the protein storage vacuoles (protein bodies). Its native molecular weight has been estimated to be 43 to 50 kD by gel filtration experiments. The inhibitor is composed of subunits of Mr 15,000 to 18,000, and it has been proposed that it is either a trimer or a tetramer of identical polypeptides or different polypeptides. The inhibitor binds to animal α-amylases at a pH optimum of 5.6, forming a stable 1:1 (molar ratio) complex. (Moreno et al., 1990)

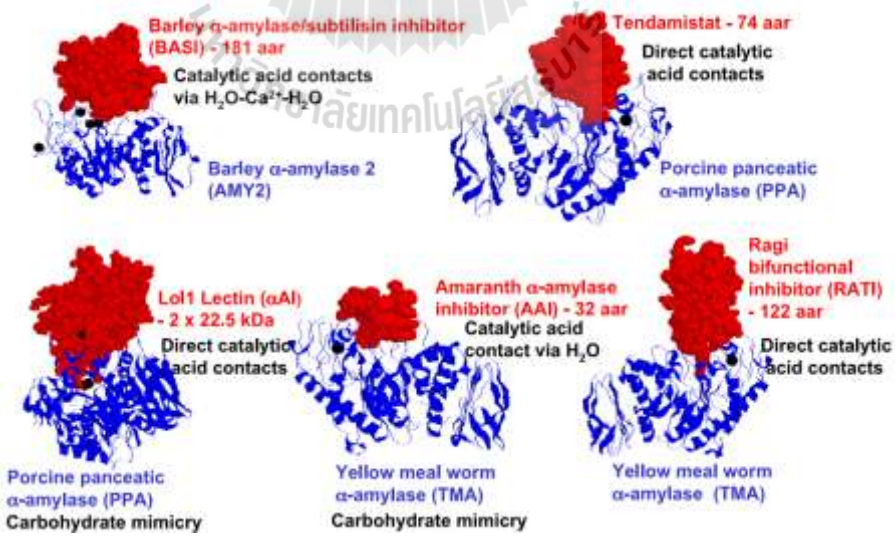

Fig. 1.1 Diversity in α-amylase/α-amylase inhibitor complexes as shown by examples on all structure-determined types.

Source : Svensson et al. (2004)

Upon substrate binding, the ‘‘flexible loop’’ moves in toward the saccharide, thus reducing the cleft breadth. In contrast, when complexed with α-AI, the same loop moves out toward the solvent, pushed away by the inhibitor as a result of the tight-binding inhibition process. This movement is accompanied by the readjustment of the surrounding regions; in particular, the loop region including residues 351–359 in domain A. The side chain of the catalytic residue Asp300 shows the same orientation in both the free structure of PPA and the α-AI–PPA complex structure (instead of its substrate-induced conformation), suggesting that Asp300 adopts this functional position when a sugar unit is bound at subsite -1(Payan, 2004).

In summary, the inhibitor α-AI completely blocks the substrate-reducing end of the enzyme cavity and prevents access to the other end via a steric hindrance process. The inhibitor triggers substrate ‘‘mimetic’’ interactions with the binding subsites on the enzyme and all the catalytically competent components of the enzyme are targeted (Payan, 2004).

The formation of the inhibitor-enzyme complex for this class of α-amylase inhibitors is pH, time and concentration-dependent (Le Berre-Anton et al., 1997). A detailed analysis of the kinetics of inhibition of the kidney bean inhibitor αAI acting on PPA indicated a two-step non-competitive slow, tight binding mechanism with a K_d = 31 μM for the first binding step and an overall K_i =30 pM. In this PPA/αAI complex the active site of the enzyme was available for catalysis. A recent study of the same system demonstrated that two inhibitor binding sites were present both on free PPA and in a PPA–substrate complex. The mechanism of inhibition was found to be mixed non-competitive and the formation of both the EI and ESI complexes was slow and required preincubation for efficient inhibition. A chimera of αAI1 that

contains an α,a β, and a γ-subunit, of which the latter corresponds to a subunit in an α-amylase inhibitor-like protein, which is an evolutionary intermediate between arcelin and αAI1, was without inhibitory activity. (Svensson et al., 2004)

1.2 Research objectives

1. This study designed to discover a suitable method for white kidney bean extraction from white kidney bean cultivate in Thailand.

2. This study focuses on the modification of several white kidney beans extraction methods, to acquire yields and highest α-amylase inhibitory activity.

3. This study focuses on the optimal extraction condition for white kidney bean extract preparation at which to acquire highest α-amylase inhibitory activity, published.

4. To study stability of white kidney bean extract in *in vitro* digestion.

1.3 Research hypothesis

White kidney bean extract cultivate in Thailand have the highest α-amylase inhibitory activity.

1.4 Scope and limitation of the study

White kidney bean extract will be analyzed for α-amylase inhibitory activities and stability in *in vitro* digestion.

1.5 Expected results

1. To study optimal extraction condition for white kidney bean extract preparation.

2. To produce white kidney bean extract preparations to may be used as food ingredient and neutraceutical product.

CHAPTER II

LITERATURE REVIEW

2.1 White kidney bean (*Phaseolus vulgaris*)

White kidney bean or *Phaseolus vulgaris* L. is a member of the Leguminosae, tribe Phaseoleae, subfamily Papilionoideae. Cultivated forms are herbaceous annuals, which are determinate or indeterminate in growth habit. *Phaseolus vulgaris* L. was originally a crop of the New World, but is now grown extensively in all major continental areas. Its production spans from 52°N to 32°S latitude. It is a major source of dietary protein throughout both Latin America and Eastern Africa, but per capita consumption is declining as population increases outdistance production (Graham and Ranalli, 1997). Archeological investigations showed that common beans originated on the American Continent, specifcally in southern United States, Mexico, Central America, and the northern part of South America. The conditions under which this annual, predominantly self-pollinated legume is grown are extremely variable. While production tends to be centered on smaller holdings, the cropping system used can vary from the highly-mechanized, irrigated, and intensive production of monoculture bush beans, to complex associations of indeterminate or climbing beans with corn, other cereals, sugar cane, coffee or plantain. Technical inputs in such multiple-cropping systems are often limited, with the result that crop yields can range from less than 500 kg ha^{-1} in parts of Latin America and Africa to as much as 5000 kg ha^{-1} under experimental conditions. The diversity of conditions under which beans are grown,

coupled with highly-specific local preferences for particular seed types or colors have complicated attempts at bean improvement. As a result, greatest progress has been in breeding for the resolution of disease, insect and nutritional constraints, with only limited. The present review considers the origins of *P. vulgaris,* the cropping systems used for bean production, the agronomic and biological factors which most limit crop yield, and the traits usually considered in bean improvement programs. It will also consider the impact of recent molecular advances to crop improvement on the prospects for the improved production of this important grain legume (Graham and Ranalli, 1997). Archeological investigations showed that *Phaseolus vulgaris* originated on the American Continent, specifically in southern United States, Mexico, Central America, and the northern part of South America. In particular, the species *P. vulgaris* was introduced into Europe in the sixteenth century and since then it has become a very important crop in many regions of the world (Carai et al, 2009).

White kidney bean are rich in dietary fibre and resistant starch fraction (Soral-Śmietana & Krupa, 2005). Substantial quantities of starch not hydrolysed in the small intestine reach the large bowel and can be fermented in the colon (Gordon *et al.*, 1997). Cassidy *et al.* (1994) suggest a strong negative correlation between the starch intake and the risk of colorectal cancer. These authors hypothesized that it is the resistant starch which provides the protection.

In comparison with cereals, in which the content of proteins varies from 5 to 15%, bean seeds are a valuable source of proteins, containing from 17 up to 39% d.m. (Bressani, 1993; Krupa & Soral-Śmietana, 2003; Soral-Śmietana *et al.,* 2003). Most of them are devoid of any catalytic activity nor play any structural role in the cotyledonary tissue. These proteins, termed as storage proteins, are stored in

membrane-bound organelles, storage vacuoles or protein bodies, in the cotyledonary parenchyma cells, survive desiccation in seed maturation and undergo proteolysis at germination, thus providing free amino acids, as well as ammonia and carbon skeletons to the developing seedling.

2.2 α-Amylase

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) is an endo-acting enzyme that are hydrolases that catalyzes the cleavage of internal glycosidic bonds between α-D-glucopyranose residues of polymers in starch and structurally similar poly- and oligosaccharides and release products with an α-D-configuration that can be absorbed. The enzymes requiring calcium as a cofactor are stimulated by chloride, bromide and fluoride and are inhibited by cadmium, copper, zinc and lead. The optimum pH range is 6.5-8.0. The enzymes are produced by a diverse variety of organisms including plant, animal, bacterial and fungal sources. Two kinds of α-amylases are produced by many mammals, salivary α-amylase from the parotid gland and pancreatic α-amylase from the pancreas which are used in the initial steps of degradation of dietary starch (Lee et al., 2002; Chokshi, 2007). The digestion of food starch begins with salivary α-amylase in the mouth, stops by the low pH of gastric juice in the stomach, the food bolus from the stomach is neutralized in the small intestine and the starch is completely digested by an α-amylase secreted from the pancreas (Yoon and Robyt, 2003). Carbohydrate normally constitutes the main component of the human diet and starch accounts for most of the ‘carbohydrate pool’. The cleavage of starch by a-amylases constitutes the first step in the enzymatic degradation of polysaccharides which is essential in carbohydrate assimilation (Payan, 2004).

The human α-amylase is classical calciumcontaining enzyme composed of 512 amino acids in a single oligosaccharide chain with a molecular weight of 57.6 kDa (Whitcomb and Lowe, 2007). There are five α-amylase genes clustered in chromosome 1, at location 1q21, in humans. Three of them code for salivary R-amylase, AMY1A, AMY1B, and AMY1C, and the other two genes AMY2A and AMY2B are expressed in the pancreas (Groot et al., 1988, Gumucio et al., 1988). Human salivary and pancreatic α-amylases share a high degree of amino acid sequence similarity with 97% identical residues overall and 92% in the catalytic domains (Ramasubbu et al., 1996, Brayer et al., 1995). The amylase presents a three-dimensional structure capable of binding to substrate and, by the action of highly specific catalytic groups, promote the breakage of the glycoside links (Iulek et al., 2000). The protein contains 3 domains: A, B, and C. Domain A, which has a (β/α)8 barrel fold, constitutes the catalytic core domain. It contains about 280–300 residues. The catalytic triad (Asp, Asp, Glu) is present in domain A (Van der Maarel, 2002). The B domain is inserted between A and C domains and is attached to the A domain by disulphide bond. The C domain presents a β sheet structure linked to the A domain by a simple polypeptide chain and seems to be an independent domain with unknown function. The active site (substratebinding) of the α-amylase is situated in a long cleft located between the carboxyl end of both A and B domains. The calcium (Ca2+) is situated between A and B domains and may act in stabilizing the three-dimensional structure and as an allosteric activator. The substrate-binding site contains 5 subsites (-3 -2 -1 +1 +2) (Brayer, 2000). α-Amylase catalyze the hydrolysis of starch via a double displacement mechanism involving the formation and hydrolysis of a covalent β- glycosyl enzyme intermediate by using active site carboxylic acids for it (Rydberg,

2002). The residues, in particular, Asp197, Glu233, and Asp300 were described to function as catalytic residues. Probably, Asp197 acts as nucleophil that attacks the substrate at the sugar anomeric center, forming a covalently bound reaction intermediate. In this step, the reducing end of the substrate is cleaved off the sugar skeleton. In a second step a water molecule attacks the anomeric center to break the covalent bond between Asp197 and the substrate, attaching a hydroxyl group to the anomeric center. In both steps Glu233 and Asp300 either individually or collectively act as acid/base catalysts. As a consequence, the active site of human α-amylase consists of several major binding subsites identified through kinetic studies. The same studies show that the "-1", "-2", and "-3" pocket is the core of the catalytic reaction (Brayer, 2000).

Fig. 2.1 Diagrams of the α-amylase (PPA) structure. The three domains are shown: domain A is colored red; domain B, yellow; domain C, purple. The calcium ion (blue sphere) and the chloride ion (yellow sphere) are also shown in the immediate vicinity of the catalytic center.

Source : Qian et al. (1997)

2.3 Alpha-amylase inhibitors (αAI)

Alpha-amylase inhibitors (αAI) are compounds that inactivate some α-amylases by forming enzyme-inhibitor complexs, as part of natural defense mechanisms. The presence of α-amylase inhibitors has also been reported in wheat, in millet, sorghum, maize, barley, rye, mangoes, peanuts, bajra, *Colocasia esculenta* tubers and acorns (Marshall and Lauda, 1975; Yamaguchi, 1993; Le Berre-Anton et al., 1997; Gibbs and Alli, 1998; McEvan et al, 2010; Pusztai et al., 2010). Proteinaceous α-amylases inhibitors can have different polypeptides scaffolds and can be grouped by their tertiary structures into six classes: lectin-like, knottin-like, cereal-type, Kunitz-like, g-purothionin-like and thaumatin-like (Payan, 2004).

α-Amylase inhibitor (αAI) in beans (*Phaseolus vulgaris*) has three different isoforms αAI-1, αAI-2 and αAI-3 (or αAI-L). αAI-1 and αAI-2 differ in their specificity towards α-amylases, despite their high degree of similarity in amino acid sequence. αAI-1 found in cultivated beans inhibits porcine pancreatic α-amylase (PPA). αAI-2 is found in some wild bean accessions. αAI-L is inactive towards all α-amylase tested so far and most probably corresponds to an evolutionary intermediate among plant defense proteins (phytohemagglutinins, arcelins and the active α-amylase inhibitors). (Lee et al., 2002; Guzman-Partida et al., 2007; Obiro et al., 2008)

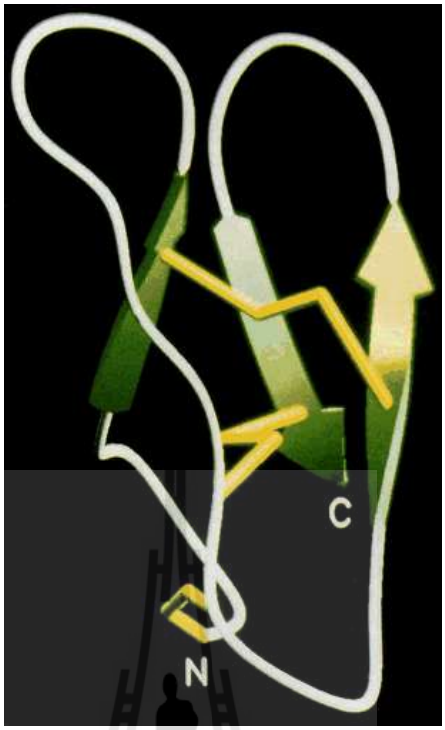

Fig. 2.2 The schematic model of the AAI structure. Disulfide bridges are shown in yellow, β-sheets are green, irregular conformations are shown in white.
Source : Chagolla-Lopez et al. (1994)

The α-AI1 is a typical bean lectin, which is synthesized in the rough endoplasmic reticulum, modified in the Golgi body through removal of a signal peptide and N-glycosylation, and transported to the protein storage vacuoles where it is proteolytically processed. SDS-PAGE, used for microsomal fractions, shows that Mr 30000–35000 fractions are associated with endoplasmic reticulum, while 14 and 19 kDa are associated with Golgi body and storage vacuoles. The a-AI1 is detectable 17 d after pollination in the cotyledons and axis of the plant seed. The amounts increase to a constant maximum after 28 d until maturity, although the amount on a dry basis decreases slightly during drying (Obiro et al., 2008).

The main interactions with PPA and HPA occur directly at the V-shaped depression of the α-amylase active site, which is also the binding site for acarbose. The inhibition process is very similar for both enzymes. In both α-AI1–HPA and α-AI1–PPA complexes, two facing hairpin loops emerging from the h-sheet fold of the inhibitor lie fully in the active site depression forming extensive hydrogen-bonding, hydrophobic and water-bridged contacts with the active site residues of the enzyme. At the heart of the substrate-binding site, two tyrosine residues (Tyr37 and Tyr186) protruding from the extremity of the two hairpin loops of α-AI combine to provide interactions with the catalytic residues, nucleophile and acid catalyst of the enzyme (Asp197 and Glu233, respectively). The interactions occurring in the region of subsites -1, + 1, + 2 are highly conserved in the complexes between carbohydrate or proteinaceous inhibitors and pancreatic α-amylases (PPA and HPA). Hydrophobic interactions also occur between the substrate's surface and the hydrophobic residues lining the entrance of the cleft (subsite -2, -3). These interactions are followed by protein–protein interactions involving areas further away from the catalytic center, namely the loop 303–312, the loop at position 237–240, the loop 347–357, and the loop 140– 150 from domain B (Payan, 2004).

Although the mechanisms of inhibition of HPA and PPA by α-AI are very similar, a close inspection of the structure of the α-AI1–HPA and α-AI1–PPA complexes showed the presence of additional hydrogen bonds between the inhibitor and domain B of the human enzyme. PPA and HPA show discrepancy in their amino acid sequences in this area which are also believed to influence the substrate specificity of the two enzymes. A different network of interactions was also observed in the loop regions 303–312 and 347–357 of domain A (Payan, 2004).

The structural changes induced in response to the binding of α-AI at the active site of the enzyme are very different from those induced by the carbohydrate inhibitor acarbose. The main displacement occurring upon the binding of the carbohydrate inhibitors involves the ‘‘flexible loop’’ (residues 303–309). An additional area that undergoes significant conformational changes is the loop extending from residues 237–240, which forms the surface edge at the substrate-reducing end periphery of the active site depression. Residues from this loop and from the ‘‘flexible loop’’ are involved in the architecture of subsite + 2. Also affected by the carbohydrate inhibitor binding process is the loop segment at position 140–150 of the domain B. Two particular acarbose-binding residues, the catalytic residue Asp300 and residue His305 undergo substantial changes. A movement of about 5 A° takes place at residue His305 which approaches the acarbose-derived ligand from the solvent side and forms a strong hydrogen bond with the residue in subsite -2. The side-chain of the catalytic residue Asp300 undergoes an induced conformational change upon substrate binding. It rotates about 60° around the Cα–Cβ bond and becomes hydrogen bonded to the acid catalyst Glu233 via an intervening water molecule (Payan, 2004).

The structure of ∝-amylase inhibitors is quite variable (Table 1) they belong to families that contain proteins of seemingly quite unrelated activity, among which are many proteinase inhibitors. Several of the structurally related proteins play a role in the stress response of plants (proteinase inhibitors, osmotin, salt-induced proteins). It is an important feature of the plant ∝-amylase inhibitors that their inhibitory activity can be species-specific. For example, members of the cereal family of amylase/protease inhibitors are active against insect ∝-amylase but do not seem to inhibit the a-amylases present in the digestive system of mammals.

Table 2.1 A classification of ∝-amylase inhibitors

Class	Source	No. of amino acid
Kunitz type	Barley, Wheat, rice	176-180
Cereal type	Wheat, barley, indian finger millet	124-160
γ-Purothionin type	Sorghum	47-48
Ragi I-2 type	Indian finger millet	95
Legume lectin type	Common beans	246
Thaumatin type	Maize	173-235
Prokaryotic type	Actinomycetes	75-120

Source : Chagolla-Lopez et al., 1994

2.4 Relationship between alpha amylase source and alpha amylase enzyme inhibitor activity

Marshall and Lauda (1975) reported that the purified α-amylase inhibitor was tested for its ability to inhibit α-amylases from a number of sources, plant, animal, fungal, and bacterial. Inhibitor (8.8 μg of protein) was preincubated with enzyme (0.05 unit) in digests (0.5 ml) containing buffer (10 mM acetate, pH 5.5), human serum albumin (0.75 mg), and calcium chloride (0.75 mg). After 30 min at 37^0C , the activity remaining was determined by addition of substrate (10 mg/ml, 0.5 ml) and incubation at 37^0C for 15 min and measurement of the amount of reducing sugars produced. Figure 4 showed the extents of inhibition of the different α-amylases are expressed relative to the activities in control digests preincubated without α-amylase inhibitor. When tested at similar concentrations, phaseolamin was found to be without inhibitory effect on sweet potato β-amylase, rabbit muscle acid and neutral α-glucosidases, and bovine pancreatic trypsin.

Phaseolamin is specific for animal α-amylases. Human salivary and pancreatic, hog pancreatic, and *Helix pomatia* α-amylase are the only enzymes which have been found to be inhibited to significant extents. The activity of plant and microbial α-amylases is unaffected by phaseolamin, although the possibility of complex formation between these enzymes and the inhibitor has not yet been ruled out. As shown in Fig. 2.3

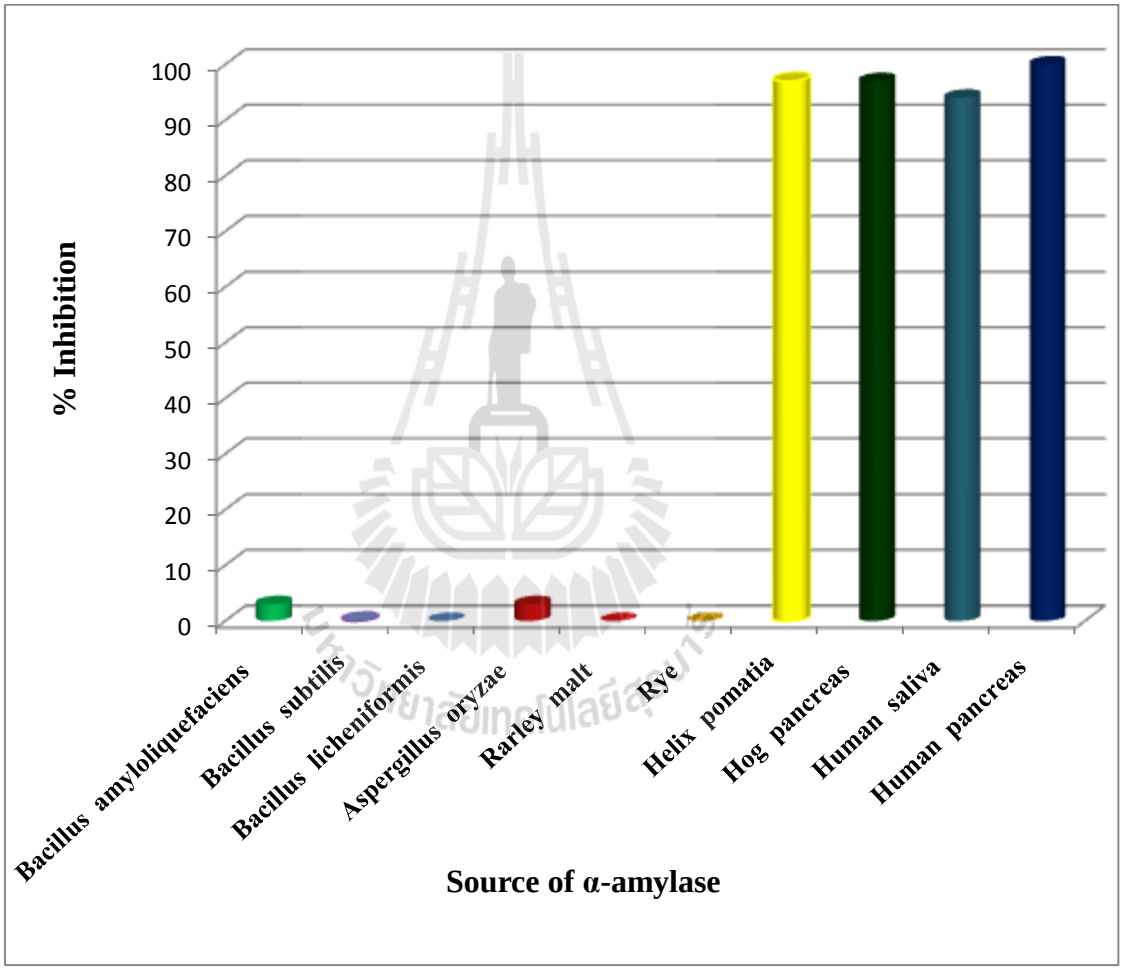

Fig. 2.3 Relationship between alpha amylase source and alpha amylase enzyme inhibitor activity

Source **:** Marshall and Lauda (1975)

2.5 Relationship between seed color of beans and α-amylase inhibitor activity

The results are arranged according to correlation between the seed coat color and α-amylase inhibitor activity. There is nonexistence of correlation between amylase inhibitor activity (AIA) and seed coat color. The inheritance of AI and seed pigments is not linked. It is possible to have similar inhibitors in different color beans and different inhibitors in same color coat beans. The results are shown in Table 2.2

Table. 2.2 Beans seed color coat and activity of α-amylase inhibitor.

color	n	activity AIU/mL	Specific activity AIU/mg of protein
white (W)	9	2.87 (2.88-3.15)	0.23 (0.14-0.33)
red (R)	14	2.85 (1.66-3.63)	0.25 (0.16-0.37)
brown (B)	20	2.82 (1.81-3.53)	0.26 (0.14-0.35)
pink (Pi)	14	2.81 (2.20-3.26)	0.21 (0.16-0.28)
black (Bl)	26	2.76 (2.01-3.57)	0.19 (0.11-0.30)
dark brown (Db)	9	2.71 (1.99-3.73)	0.25 (0.19-0.33)

Source : Iguti and Lajolo (1991)

2.6 Effect of pH on α-amylase inhibitor activity

The different pH optima reported were probably due to different incubation temperatures applied. Lajolo and Filho (1985) noted that the different pH optimal for salivary and pancreatic α-amylase activity were at 4.5 and 5.5 respectively as shown in Fig. 2.4.

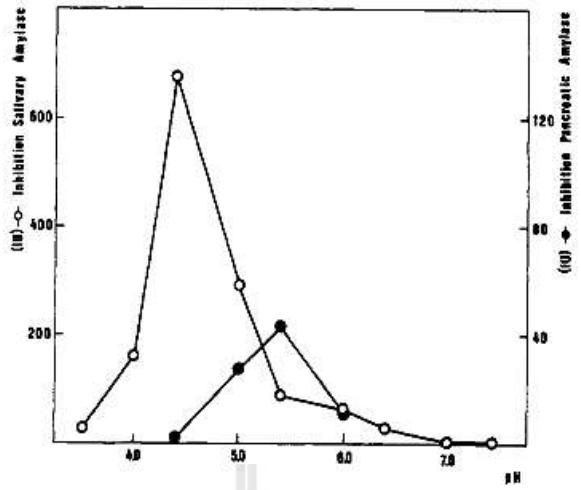

Fig. 2.4 Effect of pH on the activity of α-amylase inhibitor

Source : Lajolo and Filho (1985)

Similar experiments carried out with human pancreatic α-amylase led to identical pH values with α-AI1, Le Berre-Anton et al. (1997) demonstrated that the extent of the inhibition of PPA by α-AI1 depends on the pH value with an optimum at approximately 4.5.

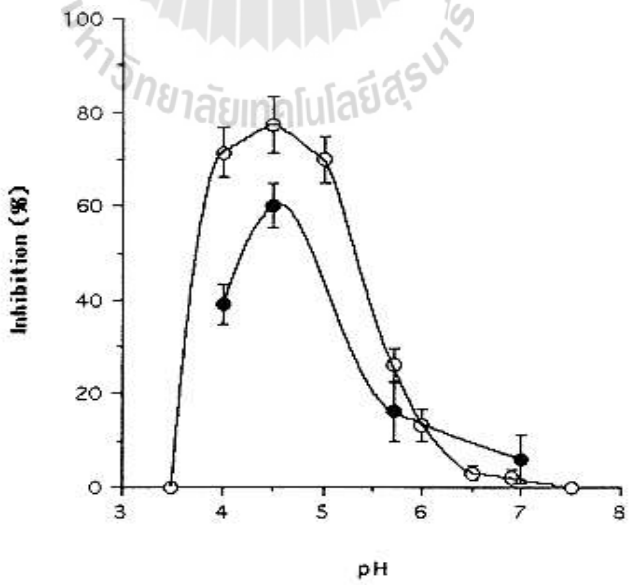

Fig. 2.5 pH dependence of inhibitor activity of α-AI1 towards porcine (○) and human (●) pancreatic amylases

Source : Le Berre-Anton et al. (1997)

Marshall and Lauda (1975) found an optimum pH of 5.5 at 37^{0}C for the inhibition of PPA by purified Great Northern white kidney bean α-AI while Powers and Whitaker (1977) reported an optimum pH of 5.0 at 30^{0}C for the inhibition of PPA by purified red kidney bean α-AI.

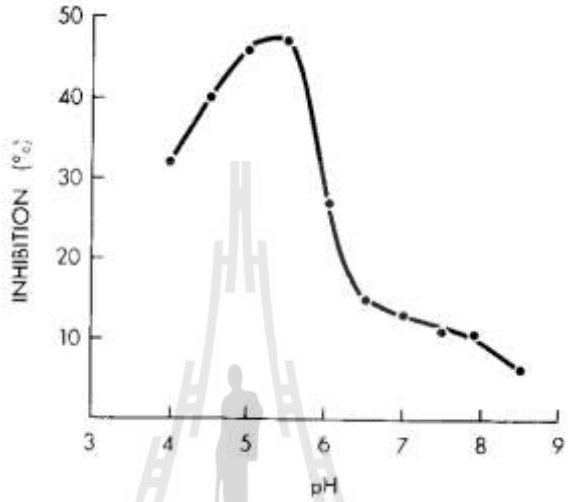

Fig.2.6 Effect of pH on inhibitory activity
Source : Marshall and Lauda (1975)

But Gibbs and Alli (1997) reported that maximal inhibitory activity was observed at pH 6.9.

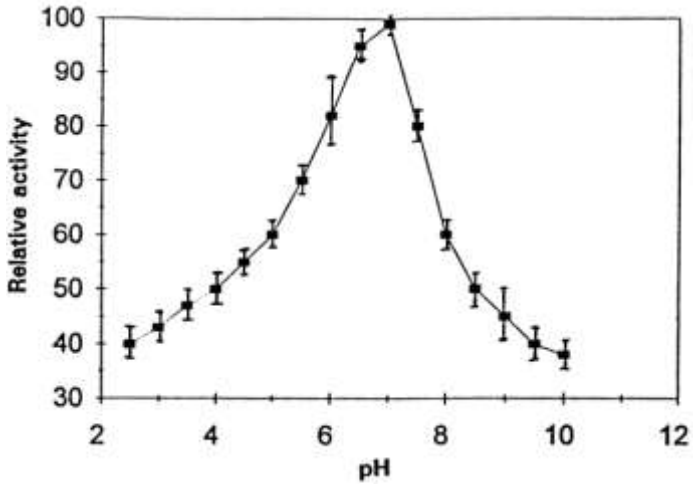

Fig. 2.7 Effect of pH on the inhibitory activity of the purified inhibitor.
Source : Gibbs and Alli (1997)

2.7 Effect of salts on α-amylase inhibitor activity

Gibbs and Alli (1997) reported the effect of various salts on enzyme/inhibitor complexation. The effect of salts on α-amylase inhibitory activity was determined by adding concentrations (10±1000mM) of sodium chloride, calcium chloride, potassium chloride and magnesium sulfate to a fixed enzyme and inhibitor concentrations whilst keeping the temperature and pH constant. Concentrations of 10±1000 mM chloride ions enhanced the binding compared to the control. A further increase in ionic concentration had no added effect on the inhibition. Magnesium or sulphate ions had no effect on inhibitory activity. Chloride ions are important for maximum activity whereas sodium increases the rate of binding as compared to potassium and calcium. Chloride ions are known to function as activators of α-amylase by modulating its action through a subtle conformational change that results in a 340 times increase in calcium binding ability. It is tempting to speculate that this allosteric effect could be involved in complex formation with the inhibitor. The results are concluded in Table 2.3

Table. 2.3 Effect of various salts on amylase inhibitory activity.

Molarity (mM)	Inhibitor activity (% of control)[a]			
	NaCl	**KCl**	**$CaCl_2$**	**$MgSO_4$**
Control[b] (0)	100	100	100	100
10	119	117	110	98
50	114	112	111	100
200	116	115	106	94
1000	110	111	113	93

[a]Salts at the final concentration indicated, were added at the beginning of the reaction. Activity was measured and was expressed as % of the control (no salts added).

[b]The specific activity of the control (0 mM) was 46,131U/mg protein.

Source : Gibbs and Alli (1997).

2.8 Temperature effect on α-amylase inhibitor activity

Lajolo and Filho (1985) studied temperature effect on the activity of the inhibitor at optimum pH of 5.4. The results showd that as temperture increased, inhibitiory activity would increased. According to Le Berre-Anton et al. (1997), the effect of various temperatures (22, 30. and 37^0C) on the inhibition of PPA at optimum pH 4.5. showed no activity of α-amylase at temperature at 0^0C. In addition, the α-amylase activity was little change temperature between 22 and 37^0C thus the temperature exhibits a moderate effect on α-AI1 activity.

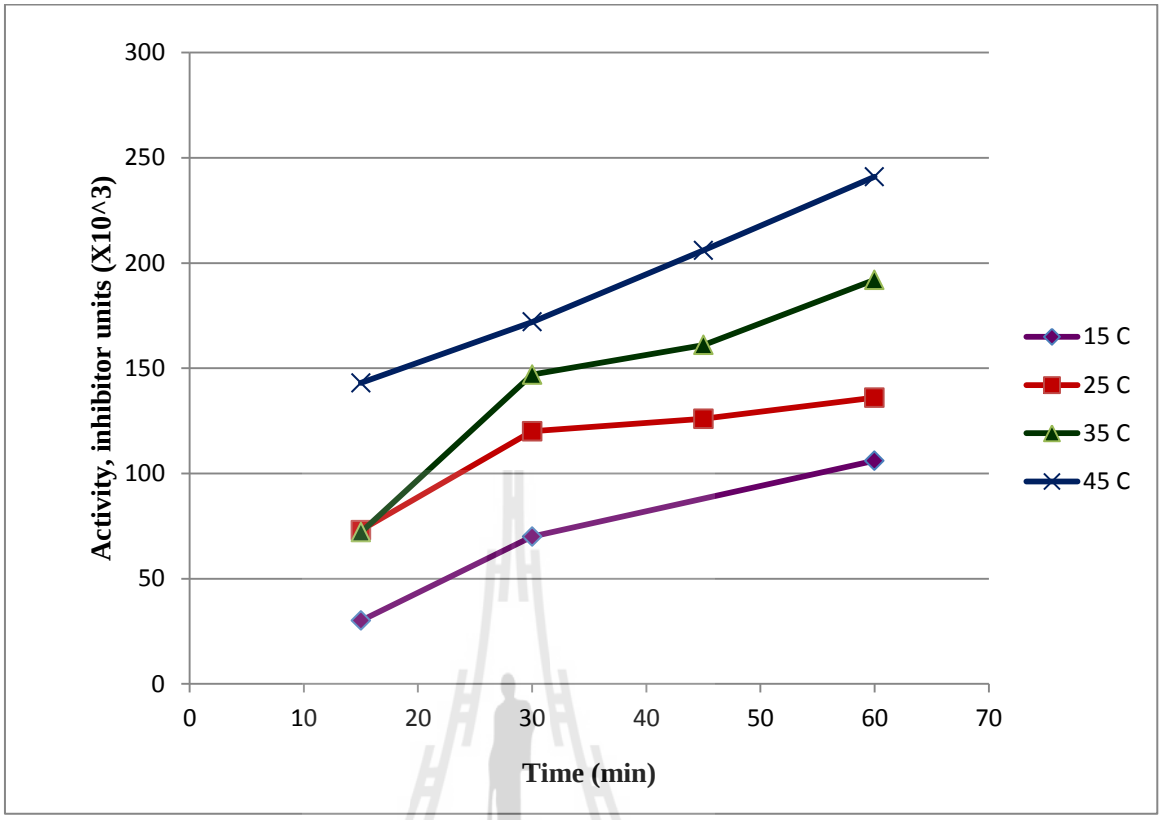

Fig. 2.8 Effect of temperature on the inhibition of porcine pancreatic amylase

Source : Lajolo and Filho (1985)

Although Marshall and Lauda (1975) also reported that there is no activity of alpha amylase inhibitor activity at temperature at 0^0C, in opposite, a 10-fold increase in activity within the temparature range at 22 and 37^0C. Le Berre-Anton et al. (1997) attributed this discrepancy to different incubation pH used. Such a discrepancy might be related to the different pH values applied in both experiments due to their enzymatic measurements were performed at pH 6.9 that is optimum pH for the porcine pancreatic α-amylase activity and not for the α-amylase inhibition acitvity. The pH and temperature effect on the inhibition activity could possibly be related to pH-induced conformational changes of the inhibitor as suggested by UV-visible spectral changes observed at different pHs.

2.9 Inhibitors of Amylase Action

Amylase inhibitors fall into one of two categories: (a) naturally-occurring proteins, that are thought to act in a defense mechanism against predators such as insects; and (b) mono- or oligosaccharides that have structural features favorable for binding relatively strongly to the active-site of the enzymes. It has been hypothysized that the oligosaccharide inhibitors mimic the transition state of the enzyme-catalyzed reaction sufficiently to bind and inhibit the enzyme. Plant protein inhibitors primarily inhibit ∝-amylases. Wheat produces an ∝-amylase inhibitor that has been known for over 50 years as Kneen's Inhibitor. Rye produces an ∝-amylase inhibitor similar to Kneen's Inhibitor, kidney beans and other legumes produce an ∝-amylase inhibitor known as phaseolamin, and oats produce a protein inhibitor that inhibits both ∝- and β-amylases.

2.10 Effect of white kidney bean extract for diabetes prevention

Although very-low-fat and lower-glycemic-index diets, regular exercise, and weight control have evident potential for prevention of type 2 diabetes, it can be anticipated that a high proportion of at-risk subjects will continue to eat whatever they want, shun exercise, and stay fat. In light of the tremendous cost of diabetes, both in terms of monetary resources and of human suffering, it would be highly desirable to have practical nutraceuticals and pharmaceuticals which such individuals could use to reduce their risk for diabetes. The range of nutraceutical compounds which might have efficacy in this regard continues to expand, and there is considerable scope for the development of products which combine effective doses of several of these

compounds. Some of these possibilities have been discussed in an earlier communication.

One reason why beans have a favorable glycemic index is that they contain ∝-amylase inhibitors (Bravo, 1998). One of these, phaseolamin, is now available commercially in a concentrated extract. Clinical studies with this preparation, as yet unpublished but cited on the internet, allegedly demonstrate that co-administration of this agent can markedly lower the glycemic index of a starchy meal. If these findings are accurate and confirmable, phaseolamin may offer an additional resource for slowing carbohydrate absorption. Although attempts to commercialize legume-derived "starch blockers" in the 1980s did not yield a useful product, it is claimed that the current product is more concentrated and thus more effective. Hopefully, credible clinical data evaluating this extract will soon be available in the refereed medical literature. Contrary to misleading representations in the lay press, there is no evidence (or reason to believe) that phaseolamin supplements will literally render dietary starch calories metabolically unavailable; any starch undigested in the intestine will likely be converted to absorbable short-chain fatty acids by bacterial action in the colon. Nonetheless, from the standpoint of diabetes prevention or control, a slowing of starch digestion could be quite worthwhile.

2.11 The effect of white kidney bean extract for body weight control

Obesity is no longer considered to be only a cosmetic problem. Studies indicate that higher levels of body fat are associated with an increased risk for the development of numerous adverse health conditions. Weight loss is increasingly recognized to have major health benefits for overweight people and also increases life

expectancy in people having obesity-related complications. While reducing dietary fat content combined with increased physical exercise was shown to be effective in preventing obesity, only one third of those trying to lose weight reported eating fewer calories and exercising more. Although weight loss and weight control drugs are becoming extremely common in today's society, the remedies provided by the diet industry have failed in the long-term maintenance of weight loss in obese patients. Moreover, it has been estimated that more than 90% of the people who lose weight by dieting return to their original weight within 2–5 years**.** Adipose tissue growth involves formation of new adipocytes from precursor cells, further leading to an increase in adipocyte size. The transition from undifferentiated fibroblast-like preadipocytes into mature adipocytes constitutes the adipocyte life cycle, and treatments that regulate both size and number of adipocytes may provide a better therapeutic approach for treating obesity. The decrease of adipose tissue mass that occurs with weight loss may involve the mobilization of lipids through lipolysis or the loss of mature fat cells through apoptosis. While development of obesity is a greater problem during middle age, elderly people can have a relative increase in body fat content accompanied by an accumulation of adipocytes in nonadipose tissues, such as uscle and bone marrow. Since marrow adipocytes inhibit osteoblast proliferation and disrupt the normal blood supply to bone tissue, treatments that inhibit marrow adipogenesis and decrease bone marrow adipocyte populations would have positive consequences for bone health. Furthermore, loss of weight in the elderly is associated with acceleration of both muscle tissue loss and bone loss, and hence, treatments that selectively remove adipocytes while sparing muscle and bone tissue could be of tremendous benefit for prevention of sarcopenia, osteoporosis and adiposity in the elderly.

Preclinical investigations have unanimously reported how the acute, repeated administration of extracts of *P. vulgaris*, as well as some of their isolated ingredients, reduced food intake, body weight, and lipid accumulation in lean and obese laboratory animals. Specifically, a study was performed to investigate the effect of a *P. vulgaris* extract mixed with a starch-enriched show on food intake and body weight in young, lean Hooded Lister rats (Grant, Dorward and Pusztai, 1993). Restricted amounts of food were made available to rats to ensure the entire supply of *P. vulgaris* extract was consumed by each rat. The results of this study indicated a significant reduction in body weight gain in rat groups consuming chow mixtures containing 20 and 40 mg/die *P. vulgaris* extract. The extract used in this study had a high content of α-amylase inhibitors, suggesting that the possible mechanism of action underlying the reducing effect produced by this *P. vulgaris* extract on body weight gain was constituted by inhibition of the pancreatic enzyme α-amylase, hampering starch metabolism and reducing feed efficiency (ie, food was less efficaciously converted into energy and, in turn, into body mass). Notably, the reduction in body weight gain secondary to exposure to the *P. vulgaris* extract was associated to a decrease in body content of lipids. Similar data were generated by a previous study, in which rats were fed with chow containing α-amylase inhibitors from *P. vulgaris*; rats displayed a decrease in body weight gain and lipid accumulation (Maranesi et al, 1984).

Two studies evaluated the effect of prolonged (700 to 800 consecutive days) exposure to a starch-enriched diet containing a *P. vulgaris* preparation. One of these two studies was designed to ensure that rats exposed to the 90 g/kg kidney bean-based diet and pair-fed control rats (a) weighed approximately 100 g at the start of the

experiment and (b) entirely consumed a fixed daily supply of food (resulting, in the treated rat group, in the consumption of the full daily dose of *P. vulgaris* extract) (Grant et al, 1995). As shown in Figure 2.9, feed efficiency (defined as the body weight gain over the amount of food intake) was largely lower, especially over the first 3-month period, in *P. vulgaris* extract-treated rats than in control rats. Additionally, a significant reduction in body content of lipids was observed throughout the study in the rat group exposed to the *P. vulgaris* extract-containing diet when compared to the rat group exposed to the *P. vulgaris* extract-free diet (Grant et al, 1995). In the second study, control rats (exposed to a *P. vulgaris* extract-free diet) had a mean body weight gain of approximately 660 g; conversely, rats consuming the diet including the *P. vulgaris* extract displayed a mean body weight gain of approximately 470 g.(Grant, Dorward and Pusztai, 1993)

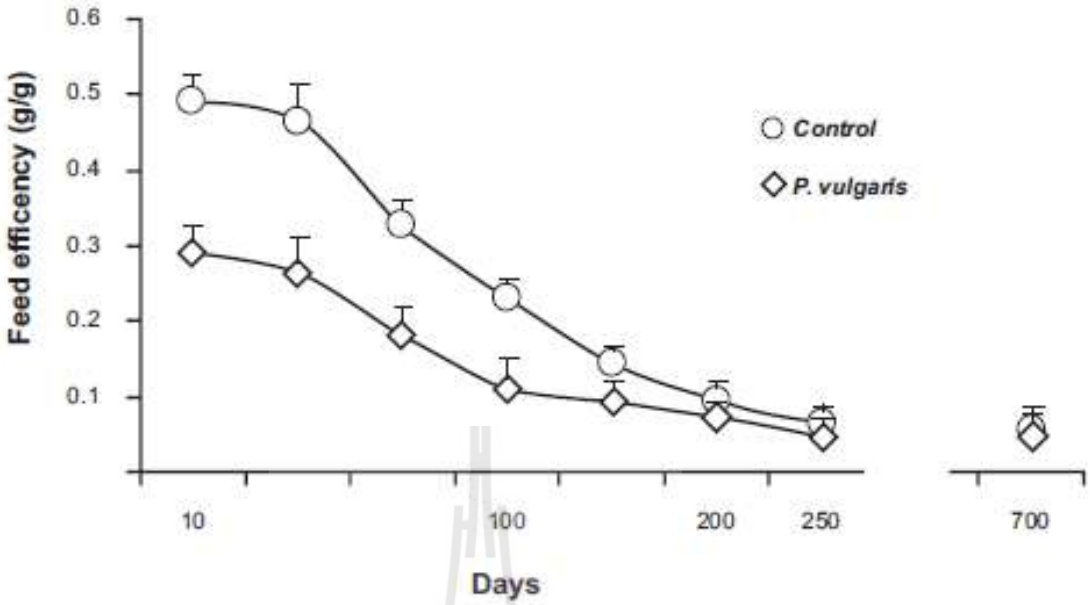

Fig. 2.9 Reducing effect of the repeated (10 consecutive days) ingestion of a *Phaseolus vulgaris* extract, mixed – at the concentrations of 0%, 0.5%, 1% and 3% – to a starchenriched diet, on daily food (top panel) and water (center panel) intake, as well as changes in body weight (expressed as percent of baseline) (bottom panel) in Wistar rats.

Source : Carai et al. (2009)

2.12 Stability

Obiro, Zhang and Jiang (2008) reported stability of the *Phaseolus vulgaris* Alpha-Amylase Inhibitor (a-Al). The inhibitor was purified to 0.09% w/w of seed flour using pH fractionation, alcohol precipitation (75%), DEAE-Sepharose CL-6B and Sephacryl S-200 chromatography. The interactive effect of pH (A), temperature (B) and time (C) on residual inhibitory activity was modeled using Response surface methodology with the Box-Behnken design. Intrinsic fluorescence and ANS-assisted surface hydrophobicity indicated activity loss is accompanied with tertiary structural

unfolding. Chaotrophic salts at high (1.0 M) and kosmotrophic salts at low (0.1-0.01 M) concentration stabilized the inhibitor in the order CH_3Coo^- > Cl^- >Br^- >I^-> SCN^- and vice-versa, respectively.

The variation of the inhibitory activity as affected by pH, temperature and heating time as would occur in different food systems however has, to the best of our knowledge, never been assessed. In addition no attempt has been made to investigate heat treatment related aspects such as effect of salts and polyols on its stability and the molecular conformational changes related to its inactivation.

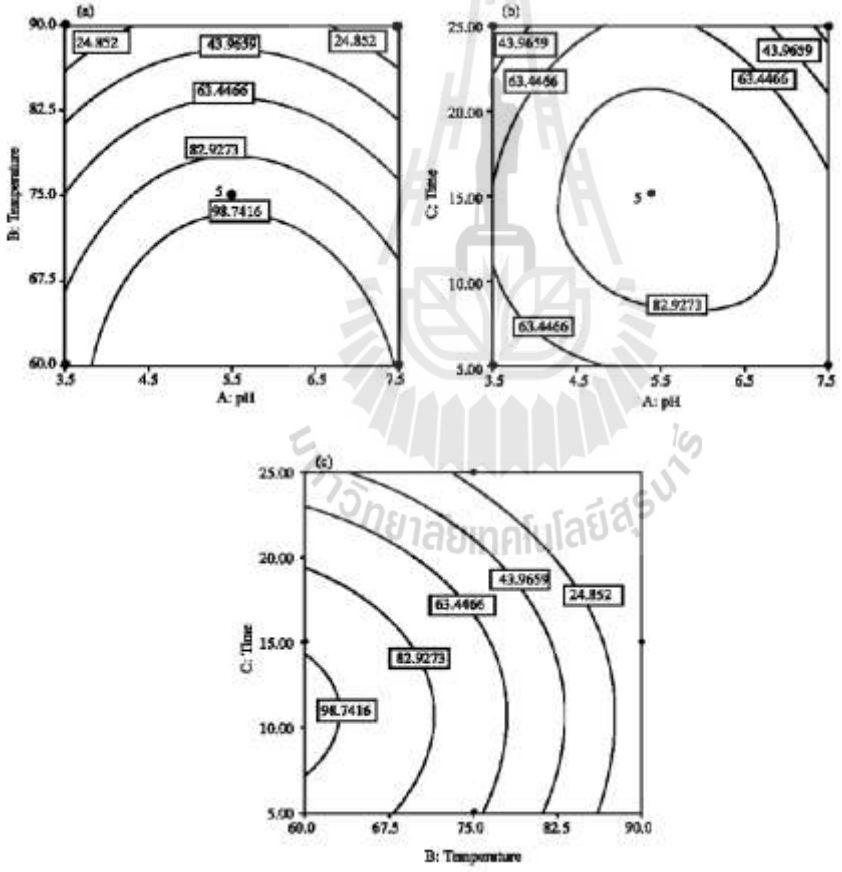

Fig. 2.10 Contours showing influence of (a) temperature-pH, (b) time-temperature and (c) pH-time interaction on residual *Phaseolus vulgaris* ∝-amylase inhibitor (∝-AI) inhibitory activity

Source : Obiro, Zhang and Jiang (2008)

The variables pH, temperature, (pH-temperature), (pH -time), $(pH)^2$, (temperature) ^{2}, (time) ^{2},$(pH)^2$ (time) were found significant The variables pH (A), Time (C), pH-Temperature (AB) and Temperature-Time (BC) had probability> F values of0.0056, 0.159, 0.171 and 0.0655, respectively, with the remaining having less than 0.0001. The coefficients showed that the linear terms of pH and temperature had a positive effect on the residual inhibitory activity. The interactive influence of the heating temperature, time and pH on the retention of inhibitory activity are illustrated in Fig. 2.10 a-c. On heating the inhibitor from 60-90oC (Fig. 2.10a), the residual activity decreased to <25% for high (7.5) and low pH (3.5), while <44.66% was retained at medium pH (5.5). In addition, the inhibitor showed maximum stability at pH 6.02. This indicated that the inhibitor would be relatively more stable in medium pH food systems than in low (3.5) like some fruit citrus juices or high pH foods. At constant temperature (Fig. 2.10b) for all pH levels the activity initially increased on heating then decreased, for example at pH 5.5 the activity increased rapidly to a max >83.336 in 10-20 min then decrease thereafter. Therefore, application of the inhibitor before heat treatment, maybe feasible in system like pasteurization.

2.13 Toxicological studies

Harikumar et al., (2005) showed no mortality or significant toxicity following oral (gavage) administration of single doses up to 5 g/ kg bw or multiple doses (90 days) up to 1 g/kg bw/day of the Phase 2 white kidney bean extract alone. These data indicated that Phase 2 white kidney bean extract was not toxic at several times higher than the typical manufacturer recommended dose of 1500 mg/day. No impairment in hepatic, renal, or hemopoetic function was observed in this investigation. In clinical

investigations, Phase 2 white kidney bean extract has shown potential usefulness in the treatment of obesity and hypertriglyceridemia (Udani et al., 2004).

Chokshi (2007) reports the findings of a 28-day oral toxicity study in rats of Phase 2 white kidney bean extract, a standardized extract derived from the common white kidney bean (*Phaseolus vulgaris*), which has been shown to have a-amylase-inhibiting activity. In order to establish safety, eighty male and female Sprague-Dawley rats (10 animals/sex/group) received Phase 2 white kidney bean extract via oral gavage at doses of 0, 625, 1250, and 2500 mg/kg (7 days/wk) for a period of 31 (males) or 32 (females) days. There were no mortalities, clinical signs, body weight or nutritional effects, gross alterations, clinical or histopathological alterations that were considered attributable to test substance administration. Under conditions of this study and based on toxicological endpoints evaluated, the no-observed-adverse-effect level (NOAEL) of Phase 2 white kidney bean extract was judged to be 2500 mg/kg/day in each sex for administration by oral gavage of a standardized white kidney bean extract, Phase 2 white kidney bean extract for 28 days.

2.14 In vitro and in vivo studies on the digestibility

The in vitro susceptibility to enzymatic hydrolysis of affinity-isolated G1, the major storage protein of the bean, has been examined. The extent of hydrolysis of G1 by a number of enzymes was less than that of native bovine serum albumin under similar conditions. Sequential treatments with different enzymes resulted in more complete hydrolysis. Discontinuous SDS gel electrophoresis of G1 after exposure to trypsin confirmed the susceptibility of the molecule to tryptic hydrolysis and indicated the presence of a number of extremely trypsin-labile peptide bonds. The existence of a

number of relatively large trypsin-resistant peptides in G1 has also been observed. The effects of heat treatment on G1 suggest that there may exist some conformational constraints on hydrolysis of the native molecule. The concentration of tannins in different seed lines did not correlate with the in vitro susceptibility to hydrolysis of the affinity-isolated protein but added tannins readily decreased the in vitro susceptibility to hydrolysis. (Romero and Ryan, 1978).

Fraction G1, the major storage protein of the navy bean *(P. vulgaris),* was subjected to in vitro digestion with pepsin, trypsin, or chymotrypsin. Based on measurements of the release of free amino groups, G1 appeared to be markedly resistant to digestion unless subjected to heat treatment. Molecular weight determinations by sedimentation equilibrium and amino acid analysis, however, indicated that G1 had in fact undergone limited proteolysis and a reduction in size, from 140,000 to 120,000, as a result of exposure to either trypsin or chymotrypsin. Disc gel electrophoresis in the presence of sodium dodecylsulfate also revealed significant changes in subunit composition. Native G1 was composed of two subunits, 43,000 and 49,000, in a ratio of 1: 2, whereas trypsin or chymotrypsin-modified G1 had subunits of 22,500 and 30,000, in a ratio of 4 : 1. The true digestibility of native G1 as measured in rats was 57% compared with 92% after heat treatment. Although virtually devoid of trypsin inhibitory activity, G1 caused a significant enlargement of the pancreas, an effect which was partially overcome by heat treatment. It is concluded that a major factor contributing to the poor nutritive value of unheated navy beans is the marked resistance of its major storage protein to intestinal proteolysis(Liener and Thompson, 1980).

2.15 Response surface methodology

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses (Box and Draper, 1987), and then migrated into the modelling of numerical experiments. The difference is in the type of error generated by the response. In physical experiments, inaccuracy can be due, for example, to measurement errors while, in computer experiments, numerical noise is a result of incomplete convergence of iterative processes, round-off errors or the discrete representation of continuous physical phenomena. In RSM, the errors are assumed to be random.

The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods (e.g. finite element method or CFD analysis) and their associated numerical noise. For example, in the case of the optimization of the calcination of Roman cement described in Section 6.3, the engineer wants to find the levels of temperature (x_1) and time (x_2) that maximize the early age strength (y) of the cement. The early age strength is a function of the levels of temperature and time, as follows:

$$y = f(x_1, x_2) + \varepsilon \qquad (2.1)$$

where ε represents the noise or error observed in the response y. The surface represented by $f(x_1, x_2)$ is called a response surface. The response can be represented

graphically, either in the three-dimensional space or as contour plots that help visualize the shape of the response surface. Contours are curves of constant response drawn in the x_i , x_j plane keeping all other variables fixed. Each contour corresponds to a particular height of the response surface.

One of the main objectives of RSM is the determination of the optimum settings of the control variables that result in a maximum (or a minimum) response over a certain region of interest, R. This requires having a 'good' fitting model that provides an adequate representation of the mean response because such a model is to be utilized to determine the value of the optimum. Optimization techniques used in RSM depend on the nature of the fitted model. For first-degree models, the method of steepest ascent (or descent) is a viable technique for sequentially moving toward the optimum response.

Generalized response surface model is given below:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_{ij}$$

Where, Y is response, which is calculated by the model; β_0 is a constant; β_i, β_{ii} and β_{ij} are linear, squared and interaction coefficients, respectively. According to Myer (1995), R^2 should be at least 0.80 for a good fit of a model. Larger values of absolute t-value and smaller values of *p*-value show that the variables would be more significant ($p \leq 0.05$). The terms found to be statistically non-significant ($p > 0.05$) were removed and experimental data was refitted to only significant ($p \leq 0.05$) factors to obtain the final reduced model. Experimental design, data analysis and optimization procedure were carried out using Design-Expert® software (Version 6.0.10, Stat-Ease, Inc., Minneapolis, MN).

2.16 Extraction of proteinaceous α-amylase inhibitor

Proteinaceous α-amylase inhibitors have been extracted from plant material with two methods including heat and non-heat extraction. Heat extraction : Sasikiran et al. (2002) extracted proteinaceous inhibitors from sweet potato by homogenizing in 0.01 M sodium phosphate buffer pH 8.0 (ratio 1 : 5 w/v) in the presence of 1.0% polyvinyl pyrrolidone (PVP). The native proteases were inactivated by heating at 70°C for 10 min and the precipitated proteins were removed by centrifugation at 1,000 x g for 10 min. To optimize the recovery of proteinaceous inhibitor from the leaves and stem the crude extract was treated with 5% TCA. After the removal of the precipitated proteins by centrifugation at 5,000 x g, the pH was rapidly brought back to 8.0 and dialyzed overnight against in 0.01 M sodium phosphate buffer pH 8.0. Iulek et al. (2000) extracted proteinaceous inhibitor from rye seeds in 1,000 ml of 70% ethanolic solution (v/v) with continuous stirring for 3 hours. The crude extract was filtered and centrifuged at 9,400 x g for 1 hour yielding a clear supernatant. The precipitate was discarded and supernatant was heated for 1 hour at 70°C, followed by another centrifugation step at 9,400 x g for 1 hour to eliminate the coagulated proteins, including the endogenous amylase. The remaining supernatant was extensively dialysed against 20 mM phosphate buffer pH 6.9 and further centrifuged at 9,400 x g for 1 hour. The supernatant was used for the inhibitor characterization. Grant et al. (1995) extracted proteinaceous inhibitor from various seeds available in Europe with 0.02 M sodium phosphate buffer pH 6.9 containing NaCl (9 g/litre-1) (1 : 5 w/v, sample to buffer ratio) for 16 hours at 4 °C and finally centrifuged at 50,000 x g for 20 min. The supernatant was heated at 70°C for 10 min, centrifuged and supernatant was used for estimation of AI content. Non-heat extraction : To obtain AI, wheat (*Triticum*

aestivum) was extracted with 0.15 M NaCl (1 : 5 w/v, sample to buffer ratio) with continuous stirring for 5 hours at 4°C for 30 min (Franco et al., 2000). AI from common bean seeds (*P. vulgaris*) were extracted with 0.15 M NaCl and 0.1%HCl (1: 5 w/v, sample to buffer ratio) with continuous stirring for 5 hours at 4°C. The mixture was centrifuged at 10,000 x g at 4°C for 30 min. The precipitate was discarded and supernatant was submitted to fractionation with ammonium sulfate (Dayler et al., 2005). Corn seeds were 4-times defatted by shaking with acetone for 15 min followed by decanting. The defatted corn flour (100 g) was extracted for its AI with 500 ml of 0.1 M acetate buffer, pH 6.0 and continuously stirred for 12 hours at 4°C. The soluble proteins were obtained by centrifuging at 30,000 x g for 20 min at 4°C. This solution was used for the inhibitor characterization (Figueira et al., 2003).

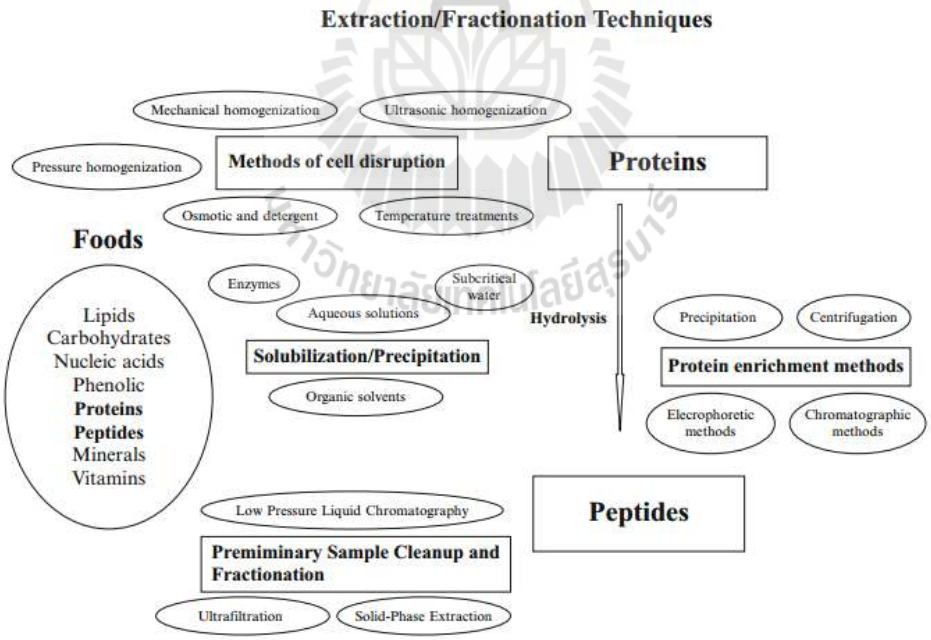

Fig. 2.11 Scheme illustrating integrated extraction and fractionation techniques for proteins and peptides employed on proteomics in foods

Source : Martínez-Maqueda et al., 2013

A wide variety of extraction and fractionation tools for proteins and peptides are available based on their physicochemical and structural characteristics such as solubility, hydrophobicity, molecular weight, isoelectric point (pI), and so on. Figure 2.11 shows an integrated view of extraction and fractionation techniques for proteins and peptides used in food proteomics studies. Generally, different technologies focused on cell disruption, solubilization/precipitation, and enrichment systems are needed to obtain the protein fraction of interest. Removal of interfering compounds (mainly lipids, nucleic acids, phenolic compounds, carbohydrates, proteolytic and oxidative enzymes, and pigments) is crucial. These procedures need to be optimized to minimize proteins´ modifications and proteolysis, as well as to be compatible with subsequent analysis.

Protein solubilization is considered one of the key steps in proteomic sample preparation procedures. It is generally employed to separate proteins in the sample selectively from different substances that may interfere in the proteomic assay (Berkelman and Stenstedt, 1998). The solubilization/precipitation process strongly affects the quality of the fi nal results and thus determines the success of the entire experiment. Taking into account the immense variety of proteins and the huge number of interfering contaminants present in food-derived extracts, simultaneous solubilization of all proteins remains a great challenge.

This method has been used to extract proteins from different tissues of cereals, legumes, and fruits. The extreme pH and negative charge of TCA and the addition of acetone realizes an immediate denaturation of the protein, along with precipitation, thereby instantly arresting the activity of proteolytic and other modifying enzymes. However, a disadvantage of TCA precipitated proteins is that they are difficult to

redissolve (Nandakumar et al. 2003). Sample solubility can be improved by using an appropriate mixture of chaotropic agents (urea or thiourea), and new efficient detergents (such as sodium dodecyl sulphate, SDS). In the last decade, the phenol extraction procedure has been widely used because of its high clean-up capacity. In contrast to its strong solvent action on proteins, phenol has little predisposition to dissolve polysaccharides and nucleic acids. However, phenol shows the disadvantages of being more time consuming than other sample precipitation procedures and of being toxic. The alcoholic extraction process after dehulling and conventional deoiling has a high efficiency of protein recovery. Aqueous alcohols (ethanol, isopropyl alcohol, butanol) are widely used on a commercial scale to remove phenolics, oligosaccharides, or inhibitors from defatted meals and seeds (Moure et al. 2006). However, as a result of the extraction with these alcoholic solvents, protein structures can be coagulated and therefore show reduced functional properties. To avoid these problems and to obtain protein concentrates or isolates with good functionality and suitable as food ingredients, mechanical and thermal treatments are applied (Moure et al. 2006 ; Barbin et al. 2011). Recently, extractions with different organic solvents, such as n-hexane, 2-methyl pentane, diethyl ether, acetone, 2-propanol, and ethanol were compared regarding effectiveness, suitability, and protein solubility of the full-fat and defatted lupin (Bader et al. 2011).

In recent years, because of the growing environmental concerns over the use of organic solvents to extract oil/protein from oil-bearing food materials, aqueous extraction is gaining attention. Water is also operationally advantageous over alcohols because it is non flammable and neither explosive nor toxic. Commercially, the production of protein concentrates (48–70% protein) or isolates (85–90% protein)

consists of an aqueous solubilization of protein and carbohydrates at acid, neutral, or alkaline pH and the selective recovery of the solubilized protein, separation, and, optionally, washing and neutralization before drying. The protein extraction yield and properties are in fluenced by the type of extraction process and by different factors such as pH, salts concentration, the ionic strength of the medium, net charge, and electrostatic repulsions (Tan et al. 2011). A number of acid and alkaline protein extraction protocols have been published from various plant and animal tissues. In the last decade, different studies have focused on evaluating the effect of extraction methods on the functional and rheological properties of proteins recovered from by-products of the meat and fish industry (Liang and Hultin 2003, Chaijan et al. 2006, Moayedi et al. 2010, Omana et al. 2010). In the case of plant proteins, the ideal extraction method is particularly challenging due to the metabolic and structural characteristics of plant tissues, including the cell wall matrix. The majority of alkaline extraction protocols are based on the so-called Osborne method (Osborne, 1924) , but each method is optimized according to the aim of the study and the type of vegetal protein source. Recent studies report the use of mainly sodium and calcium salts to extract proteins from different vegetal foods (Ghaly and Alkoaik, 2010, Nadal et al. 2011). These extraction methods are simple because the agents required are easily available. However, as a result of the degradation at high pH conditions, the protein yield is generally low. Also, the protein quality can be altered by alkaline processing due to undesirable reactions involving racemization of amino acids, formation of toxic compounds such as lysine alanine, reduction of digestibility, loss of essential amino acids, and decrease in nutritive value. Furthermore, the remaining alkali needs to be washed thoroughly from the final product, leading to generation of a large amount of

wastewater (Sereewatthanawut et al. 2008). To optimize protein precipitation recovery different strategies have been developed. Use of additives, such as TCA or carboxymethylcellulose is generally accepted (Massoura et al. 1998). Extraction and further formation of protein micelles have also been proposed (Krause et al. 2002, Green et al. 2010). This method has been demonstrated to reduce the concentration of problematic antinutritional or toxic factors, including the glucosinolates and their degradation products during canola protein extraction (Tan et al. 2011).

The use of centrifugation is one of the simplest methods used for isolation and enrichment/fractionation of proteins. Centrifugation can be used for different purposes. It can be a first step to separate different cell substructures where our proteins of interest are locally concentrated, for instance, mitochondria, membrane, or nucleus. This process involves multiple centrifugation steps and, as a result, the cellular homogenate is separated into different layers based on the molecular weight, size, and shape of each component. Afterwards, solubilization steps, as explained above, and enrichment and fractionation steps should be carried out to isolate the protein fraction from the selected layer prior to MS analysis. Apart from its use separating crude mixtures of cell components, centrifugation is also commonly used to fractionate a protein mixture into different fractions. The separation takes place based on the coefficient of sedimentation of the proteins. This coefficient is usually expressed in Svedberg units (S), and the smaller the S value, the slower a molecule moves in a centrifugal field. Separation will depend on the mass, the shape, and the protein density. Numerous examples are found in the literature using the differential coefficient of sedimentation of the proteins to carry out fractionation (Sharma et al. 2010 ; Jiang et al. 2011). The efficiency of this fractionation step can be enhanced

using gradient centrifugation, where the centrifuge tube is filled with a solution of sucrose, forming a density gradient.

2.17 Electrophoretic Methods

Electrophoresis separates mixtures of proteins based on charge, charge/mass ratio, size, or shape. This technique is mainly used as an analytical and preparative tool, especially one-dimensional separation, often employed as a pre-fractionating technique (Guttman et al. 2004; Jorgenson and Evans 2004). Often, laboratories dedicate one-dimensional gel electrophoresis (1DE) to evaluate the outcome of protein purification preceding the analysis by (2DE) (Chen et al. 2007).

Electrophoretic pre-fractionation methods include electrokinetic methodologies performed in free solution, essentially all relying on isoelectric focusing (IEF) steps. Purification using IEF is especially advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins remain in solution in their native conformation. Based on the IEF principle, different instruments have been developed such as the Roto for, a multicompartmental device with focusing chambers that allows the fractionation of volumes of sample (12–60 mL) containing micrograms to grams of protein (Hey et al. 2008). Another well-known device is the so-called “Off-Gel IEF” (Keidel et al. 2011). Upon application of an electric field perpendicularly to the liquid chamber, the current lines penetrate into the chamber and extract charged proteins from the solution into the IEF gel. In its multicompartment format, the protein fractions are separated by ranges of pI depending on their positioning over the IEF gel strip. Other instruments of interest are the Octopus, a continuous- flow device for isoelectric focusing in an upward flowing liquid curtain, and the Gradiflow, where

different pI cuts are obtained by a multistep passage through two compartments buffered at different pH values (Righetti et al. 2003). Depending on the complexity of the samples, the separated fractions can be analyzed directly by MS or in some cases they may undergo a subsequent separation step in a second dimension, generally SDS-PAGE, to separate the proteins according to their molecular weight. In the first case, the possible presence of ampholytes may imply an extra step to remove them and avoid disturbance in MS.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

Commercial white kidney beans (*Phaseolus vulgaris*) cutivar Pangda 2 were obtained from Royal project foundation, Thailand, were ground using a milling machine (Satake co., Hiroshima, Japan) to a fine powder in a mill and the resultant flour passed through a 2 mm sieve and kept in vacuum package at 4° C until used.

3.1.2 Chemical

∝-amylase from porcine pancreas Type VI-B Lipase from porcine pancreas Type II and pepsin from porcine mucosa were purchased from Sigma Co. (St. Louis, MO, USA). All other reagents used were of analytical grade. Reagents used for gel electrophoresis were purchased from Promega (Madison, WI, USA) Alcalase 2.4 L was gifted from Novozymes (Bagsvaerd, Denmark). Trypsin from hog pancreas was purchased from Biochemika (Buchs, Switzerland). All other reagents used were of analytical grade.

3.2 Proximate analysis

Dry white kidney beans powder were analysed for moisture, ash, sand, fat, crude fiber and protein by AOAC (2005) as the following ; moisture (AOAC 925.10), ash (AOAC 900.02A), sand (AOAC 900.02D), protein (AOAC method 928.08), fat (AOAC 963.15), crude fiber (AOAC 978.10).

3.2.1 Moisture

Drying to constant weight in an oven at 110°C for 24h was applied to determine moisture content of dry white kidney beans. One g of dry white kidney beans was placed into a pre-weighed can and dried in an oven at 110°C for 24 h. After removing the samples from the oven, they were placed in a desiccator to cool and then reweighed. The moisture content of the samples was calculated as:

$$\text{Moisture percentage} = \frac{(\text{A-B})\times 100}{\text{C}}$$

A = weight of moisture can + weight of sample before oven

B = weight of moisture can+ weight of sample after oven

C = weight of sample

3.2.2 Ash

The ash content of dry white kidney beans was determined by dry ashing. Approximately one g of sample was placed in a porcelain crucible in a muffle furnace at 600°C for 3h. After ashing, the samples were removed from the furnace, cooled to room temperature in a desiccator and then reweighed. The ash content was calculated as:

$$\%\text{Ash} = \frac{\text{Difference in Wt.of Ash}\times 100}{\text{Wt.of sample}}$$

3.2.3 Sand

Add 25 ml of 10% hydrochloric acid on the ash that has been burned off with mirror boil gently for about 5 minutes. Then filtered while hot through ashless filter paper and washed with water several times. Take filter paper in same cup glaze. Then it was burned in a furnace at 550 ° C for 12-18 hours or until a constant weight of the sample . Then the sample out of the furnace. Let cool in the desiccator.

Sample is weighed Analyzes per sample 3. Repeat calculate the amount of sand then the average

$$\%\text{Sand} = \frac{\text{Wt. of sand} \times 100}{\text{Wt.of sample}}$$

3.2.4 Crude protein

Crude protein is determined by measuring the nitrogen content of the feed and multiplying it by a factor of 6.25. This factor is based on the fact that most protein contains 16% nitrogen. Crude protein is determined by kjeldahl method. The method involves: Digestion, Distillation and Titration.

Digestion: weigh about 2g of the sample into kjeldahl flask and add 25mls of concentrated sulphuric acid, 0.5g of copper sulphate, 5g of sodium sulphate and a speck of selenium tablet. Apply heat in a fume cupboard slowly at first to prevent undue frothing, continue to digest for 45mins until the digesta become clear pale green. Leave until completely cool and rapidly add 100mls of distilled water. Rinse the digestion flask 2-3 times and add the rinsing to the bulk.

Distillation: Markham distillation apparatus is used for distillation. Steam up the distillation apparatus and add about 10mls of the digest into the apparatus via a funnel and allow it to boil. Add 10mls of sodium hydroxide from the measuring cylinder so that ammonia is not lost. Distil into 50mls of 2% boric acid containing screened methyl red indicator.

Titration: the alkaline ammonium borate formed is titrated directly with 0.1N HCl. The titre value which is the volume of acid used is recorded. The volume of acid used is fitted into the formula which becomes

$$\%\text{N} = \left(\frac{14 \text{ x VA x } 0.1 \text{ x w}}{1000\text{x}100}\right) \text{x}100$$

VA = volume of acid used

w= weight of sample

%crude protein = %N x 6.25

3.2.5 Crude fat

Crude fat of dry white kidney bean was determined by Soxhlet extraction using petroleum ether on a SoxtecTM 2050 auto fat extraction system. Briefly, one g of the sample was put into a Soxhlet extraction thimble and 80 ml of petroleum ether were added. The thimble was moved to the Soxtec System. Extraction was performed for 4 h. Subsequently, the solvent was evaporated off, the extraction cup was removed and placed into an oven at 110°C. After 1 hour the drying cup was removed from the oven and left to cool in a desiccator. Fat content was calculated as :

$$\%\text{Fat} = \frac{\text{Wt.of cup after extraction(g) - Wt.of cup before extraction(g)}}{\text{Wt.of sample (g)}} \times 100$$

3.2.6 Crude fiber

The organic residue left after sequential extraction of feed with ether can be used to determine the crude fiber, however if a fresh sample is used, the fat in it could be extracted by adding petroleum ether, stir, allow it to settle and decant. Do this three times. The fat-free material is then transferred into a flask/beaker and 200mls of pre-heated 1.25% H_2SO_4 is added and the solution is gently boiled for about 30mins, maintaining constant volume of acid by the addition of hot water. The buckner flask funnel fitted with whatman filter is pre-heated by pouring hot water into the funnel. The boiled acid sample mixture is then filtered hot through the funnel under sufficient suction. The residue is then washed several times with boiling water (until the residue is neutral to litmus paper) and transferred back into the beaker. Then 200mls of pre-heated 1.25% Na_2SO_4 is added and boiled for another 30mins. Filter

under suction and wash thoroughly with hot water and twice with ethanol. The residue is dried at 650 ˚C for about 24hrs and weighed. The residue is transferred into a crucible and placed in muffle furnace (400-6000C) and ash for 4hrs, then cool in desiccator and weight.

$$\% \text{ Crude fiber} = \left(\frac{\text{Dry wt of residue before ashing - wt of residue after ashing}}{\text{wt of sample}} \right) \text{x}100$$

3.3 Selection and modification extraction method

The fine powder was used for extraction by different adaptation method by ratio 1:6 as detailed in the following:

3.3.1 Mosca et al. (2008)

White kidney beans powder were extracted by stirred overnight with 10 mM phosphate buffer pH 7.2 containing 150 mM NaCl (1:6w/v) at room temperature. After centrifugation (10000×g, 35min, 10 ◦C), the supernatant was collected and the pellet was extracted twice in PBS (10mL), stirred for 2 h. at room temperature and centrifuged as above. The clear supernatants were collected, filtrated and stored at −20 ˚C

3.3.2 Marshall and Lauda (1975)

White kidney beans powder were centrifuged with sodium chloride solution 1% (1:6 w/v) at 4˚C. The homogenate was centrifuged at 10,000 g for 1 h. at 4˚C. The supernatant was filtered through cotton wool and centrifuged at 10,000 g for 1 h. at 4˚C. The clear supernatants were collected, filtrated and stored at -20 ˚C.

3.3.3 Le Berrre-Anton et al. (1997)

White kidney beans powder were extracted by stirred overnight with water (1:6 w/v) at 4˚C. The homogenate was centrifuged at 10,000 g for 15 min at 4˚C and supernatant was filtered through cotton wool. 110 µl/ml of 200 mM succinate buffer

pH 3.8 containing 10 mM CaCl was added into supernatants. The protein precipitate was removed by centrifugation at 10,000 g for 15 min at 4˚C. The clear supernatants were collected, filtrated and stored at -20 ˚C.

3.3.4 Giri et al. (1996)

White kidney beans powder were extracted by stirred for 2-2.5 h. with 0.1 M HCl (1:6 w/v) at room temperature (25˚C). The homogenate was centrifuged at 10,000 g for 45 min at 4˚C. The supernatant was filtered through cotton wool and centrifuged at 10,000 g for 45 min at 4˚C. The clear supernatants were collected, filtrated and stored at -20 ˚C.

3.3.5 Grant et al. (1995)

White kidney beans powder were extracted by stirred for 16 h. with 0.02 M Sodium phosphate buffer, pH 6.9 , NaCl 9 g/L (1:6 w/v) at 4˚C. The homogenate was centrifuged at 10,000 g for 1 h. at 4˚C. The supernatant was filtered through cotton wool and centrifuged at 10,000 g for 1 h. at 4˚C. The clear supernatants were collected, filtrated and stored at -20 ˚C.

3.4 Protein concentration

Protein concentration was determined by the Bradford method (Bradford, 1976) using BSA as a standard. Prepare a series of protein standards using BSA diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 0.1, 0.2, 0.3, 0.5, 0.7 and 1 mg BSA/mL. Also prepare serial dilutions of the unknown sample to be measured. Add 100 µL of each of the above to a separate test tube. Add 5.0 mL of Coomassie Blue to each tube and mix by vortex, or inversion. Adjust the spectrophotometer to a wavelength of 595 nm, and blank using the tube which

contains 0 BSA. Wait 5 minutes and read each of the standards and each of the samples at 595 nm wavelength. Plot the absorbance of the standards vs. their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

3.5 Enzyme assays

3.5.1 Specific inhibition activity

Specific inhibition activity was measured using the method described by Bernfeld (1955) with modifications, by measuring the residual α-amylase activity after enzyme and inhibitor were preincubated for 15 min, at 37 ˚C, in 20 mM acetate buffer pH 4.5, 20 mM $CaCl_2$, 10 mM NaCl. After the reduced starch was added and incubated for 15 min at 37 ˚C, reaction was stopped by addition of 0.8 ml of dinitrosalicylic acid reagent (DNS). 1 ml of this solution was boiled for 5 min, then cooled and diluted with 4 ml of water then the absorbance was read at 540 nm. Appropriate blanks were prepared without α-amylase also the residual activity was measured. A control without enzyme inhibitor addition was done for the specific inhibition activity calculation.

$$\text{Specific inhibitory activity} = \frac{\text{Inhibition activity}}{\text{mg of protein}}$$

3.5.2 ∝-*Amylase activity*

The ∝-amylase activity in the absence of the inhibitor was measured 100 microliters of porcine pancreatic ∝-amylase solution (10 U mL^{-1} in 20 mM phosphate buffer pH 6.9 containing 6.7mM sodium chloride) was added to 100μL of 20mMphosphate buffer pH 6.9 containing 6.7mM sodium chloride. The reaction was then started by adding 800 μL of 1% starch solution in 20mM phosphate buffer pH 6.9

containing 6.7mM sodium chloride. The reaction was allowed to proceed at 37 ◦C and stopped in ice by adding 1mL of NaOH 2N after 5min.

3.5.3 ∝-*Amylase inhibitor activity*

The ∝-amylase inhibitor activity was assayed by measuring the residual ∝-amylase activity in the presence of the sample extract containing the inhibitor. The assay was performed by adding from 25 to 100μL of sample extract to 100μL of porcine pancreatic ∝-amylase solution, in order to obtain 50% inhibition. An appropriate blank was prepared without ∝-amylase in order to correct for any endogenous amylase activity. The mixtures were brought to a total volume of 200μL with 20mM phosphate buffer pH 6.9 containing 6.7mM sodium chloride and were incubated at 37 ◦C for 45 min. After the addition of starch and incubation for 5min at 37 ◦C as described above, the reaction was stopped and the ∝-amylase activity was measured.

$$\% \text{ inhibition} = \frac{\propto\text{-amylase inhibitor activity}}{\propto\text{-amylase activity}} \text{X } 100$$

3.6 Yield of extract

$$\% \text{ yield} = \left(\frac{\text{extract wt.}}{\text{raw material wt.}}\right) \text{X } 100$$

3.7 Optimazation extraction

From Selection and modification extraction method, Mosca et al. (2008) adaptation method is best method for find optimize extraction.

Then adaptation method as detailed in the following:

White kidney beans powder were extracted with 0.05M, 0.10M, 0.15M PBS (10mM phosphate buffer pH 7.2 containing 150mM NaCl) (1:6 powder : PBS w/v) by

stirring 1, 2 and 3 hour at room temperature (25˚C). The homogenate was centrifuged at 10,000 g for 30, 45, 60 min at 4 □C twice times. The clear supernatants were collected, filtrated and stored at -20 ˚C.

One-Way ANOVA was applied to identify optimum levels of three variables of the concentration of PBS (M), extraction time (hour) and separation time (min) regarding of two responses extract yields and alpha-amylase enzyme inhibitory activity in the *Phaseolus vulgaris* extracts. The design independent and dependent variables are list in Table 3.1. Concentration of PBS (X_1), extraction time (X_2) and separation time (X_3). The experiments were designed according to the box-behnken design as shown in Table 3.1. The order of the experiments has been fully randomized. Data were analyzed by One-Way ANOVA.

Table 3.1 Uncoded and coded levels of independent variables used in the experimental design.

Symbols	**Independent variables**	**Coded levels**		
		− 1	0	1
X_1	Concentration of PBS (M)	0.05	0.10	0.15
X_2	Extraction time (hour)	1	2	3
X_3	Separation time(min)	30	45	60

.

3.8 Statistical analysis

Results are expressed as means of values ± standard deviation. Analysis of variance was used to determine the least significant differences in mean values (at $p < 0.05$) from the three replicates using the statistical software SPSS Inc., USA, version 13.

3.9 Electrophoresis

The Experion system is the ideal complement to a number of applications including sizing and quantitation of protein in white kidney bean extract and trade white kidney bean extract. Automated electrophoresis systems typically include an electrophoresis station, priming station, vortex station, computer and software for operation and analysis. The Experion system performs automated sample separation, staining, destaining, imaging, band detection, quantitation, and data analysis in as little as 30 minutes. Protein molecular weight makers (Bio-Rad Laboratories, Hercules, CA). The Experion Pro260 analysis kit makes protein separation, sizing, and quantitation fast and easy. The Pro260 kit offers the ability to analyze 10 protein samples (between 10 and 260 kD) in approximately 30 minutes. Accurate sizing is achieved with the Experion Pro260 ladder, part of the Precision Plus Protein™ family of standards.

3.10 In vitro digestion

The in vitro digestibility of native and heated G1 was assessed by measuring the extent to which the protein was degraded by treatment with pepsin, trypsin, or chymotrypsin, either singly or in combination. To a 1% solution of G1 dissolved in an appropriate buffer (0.2M KC1-HC1, pH 2, for pepsin; or 0.2M phosphate buffer, pH 7.6, for trypsin and chymotrypsin) was added a level of enzyme equivalent to 1% of the concentration of G1. Digestion was allowed to proceed at 37°C, and, at various intervals, an aliquot of the digest was removed and deproteinized with five parts of 5% trichloroacetic acid.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Proximate composition

The dried white kidney bean powder composed of moisture 11.07±0.01 %, ash 4.10±0.01 %, sand 0.01±0.00 % , fat 1.80±0.02 %, crude fiber 31.73±0.01 % and protein 20.28±0.33%. The ash and protein content are relatively close to the values reported by Barampama and Simard (1993) were 4.47% and 22.26% respectively. In opposite, the moisture and fat content are higher when compare to the values reported by Barampama and Simard (1993) were 9.19% and 1.01% respectively.

Table 4.1 Proximate composition of the dried white kidney bean

Nutrient	**% Composition**
Moisture	11.07±0.01
Ash	4.10±0.01
Sand	0.01±0.00
Fat	1.80±0.02
Crude fiber	31.73±0.01
Protein	20.28±0.01
Energy	221.36 cal/100 g

All values are means of triplicate determinations ± standard deviation.

4.2 Selection and modification extraction method

In this thesis were selected 5 extraction methods for find method that have the most specific inhibition activity which involves α-amylase inhibitor in white kidney bean extract.

Table 4.2 Yield of extract

Methods	yield (%)
Mosca et al. (2008)	14.89
Marshall and Lauda (1975)	8.95
Le Berrre-Anton et al. (1997)	10.01
Giri et al. (1996)	14.75
Grant et al. (1995)	11.97

Yield of extract from Mosca et al. (2008) adaptation method is the most. Salts can affect protein conformation to the extent that the anions or cations of the salt could be potential buffer components. When the salt concentration is much larger than that of the buffer, the salt becomes the effective buffer in the reaction.

Protein concentration in white kidney bean extracts as a result of many reasons including extraction's solvent, pH, temperature and time in extraction. α-amylase inhibitors can have different polypeptides scaffolds.

Table 4.3 Protein concentration in white kidney bean extracts

Methods	Protein concentration (mg/g of dried weight raw material)
Mosca et al. (2008)	6.49±0.09[a]
Marshall and Lauda (1975)	3.19±0.28[c]
Le Berrre-Anton et al. (1997)	1.64±0.11[d]
Giri et al. (1996)	1.42±0.09[d]
Grant et al. (1995)	3.98±0.06[b]

All values are means of triplicate determinations ± standard deviation.

Means within columns with different letter (a, b, c, d) are significantly different ($p < 0.05$).

The crude extract by Mosca et al. (2008) adaptation method showed highest protein concentration at 6.49±0.09 mg/g of dried weight raw material and Giri et al. (1996) adaptation method showed lowest protein concentration at 1.42±0.09 mg/g of dried weight raw material (Table 4.3)

Table 4.4 α-Amylase inhibitory activity in white kidney bean extracts

Methods	Specific inhibition activity (per mg of protein)
Mosca et al. (2008)	34.69±0.56.[a]
Marshall and Lauda (1975)	13.14±1.07[c]
Le Berrre-Anton et al. (1997)	22.99±1.08[b]
Giri et al. (1996)	34.54±2.73[a]
Grant et al. (1995)	10.89±0.20[c]

All values are means of triplicate determinations ± standard deviation.

Means within columns with different letter (a, b, c,) are significantly different ($p < 0.05$).

Both Mosca et al. (2008) and Giri et al. (1996) adaptation method crude extracts show highest specific inhibition activity significantly at $p<0.05$ when compare with others. (Table 4.4)

Finally, It could be concluded that Mosca et al. (2008) adaptation method or Giri et al. (1996) adaptation method showed be selected for extracts preparation focus on highest specific inhibition activity.

Specific inhibition activity related with α-amylase inhibitor in extract, if extract have more α-amylase inhibitor so specific inhibition activity is high, too. White kidney bean extract from Mosca et al., (2008) adaptation method have highest specific inhibition activity shows time of extraction is effect to protein concentration and specific inhibition activity.

4.3 Optimization extraction method

From previous experiment Mosca et al., (2008) is the best in 5 adaptation methods for find optimize extraction.

Then adaptation method as detailed in the following:

White kidney beans powder were extracted with 0.05M, 0.10M, 0.15M PBS (10mM phosphate buffer pH 7.2 containing 150mM NaCl) (1:6 powder : PBS w/v) by stirring 1, 2 and 3 hour at room temperature (25˚C). The homogenate was centrifuged at 10,000 g for 30, 45, 60 min at 4 □C twice times. The clear supernatants were collected, filtrated and stored at -20 ˚C

The yields of crude extract (Y_1), specific inhibition activity (Y_2) in white kidney beans (*Phaseolus vulgaris*) crude extracts obtained from all the experiments are listed in Table 4.5

Table 4.5 Experimental design and responses of the dependent variables to the extract parameters.

Exp. No[a].	Independent variables			Yield(%) (Y_1)	Specific inhibition activity (per mg of protein) (Y_2)
	Concentration of PBS (M) X_1	Extraction time (hour) X_2	Separation time (min) X_3		
1	0.1 (0)	1 (-1)	30 (-1)	17.97	42.49
2	0.1 (0)	3 (1)	30 (-1)	13.26	34.76
3	0.1 (0)	1 (-1)	60 (1)	17.71	34.31
4	0.1 (0)	3 (1)	60 (1)	14.61	36.89
5	0.05 (-1)	1 (-1)	45 (0)	13.25	37.01
6	0.05 (-1)	3 (1)	45 (0)	13.6	39.71
7	0.15 (1)	1 (-1)	45 (0)	16.79	17.43
8	0.15 (1)	3 (1)	45 (0)	22.88	16.97
9	0.05 (-1)	2 (0)	30 (-1)	12.54	33.96
10	0.05 (-1)	2 (0)	60 (1)	12.53	33.77
11	0.15 (1)	2 (0)	30 (-1)	22.21	17.76
12	0.15 (1)	2 (0)	60 (1)	23.32	18.09
13	0.1 (0)	2 (0)	45 (0)	14.31	34.88
14	0.1 (0)	2 (0)	45 (0)	14.18	34.59
15	0.1 (0)	2 (0)	45 (0)	14.99	34.68

[a] Experiments were conducted in a random order.

+1 = High level, 0 = Medium level,-1 = Low level

Results were statistically analyzed by Analysis of Variance. Mean analysis was performed using Duncan's procedure at $p \leq 0.05$. RSM was focused on determining regression coefficients and statistical significance of model terms. RSM helps in fitting regression models to experimental data to achieve an overall optimal region for all response variables studied.

From Table 4.5, the highest yield had shown at the condition of solvent concentration at 0.15M, extraction time for 2 hour, and separation time for 60 min. In addition, at what time data was analyzed for correlation between independent variable and yield percentage, the statically analysis showed significant at p value less than 0.01. In the studied of optimal condition to get highest specific inhibition activity to alpha amylase enzyme, the results showed that at the solvent concentration at 0.10M, extraction time for 1 hour, and separation time for 30 min well correlated with statistically significant at p value less than 0.01.

Fig 4.1 White kidney bean (*Phaseolus Vulgaris*).

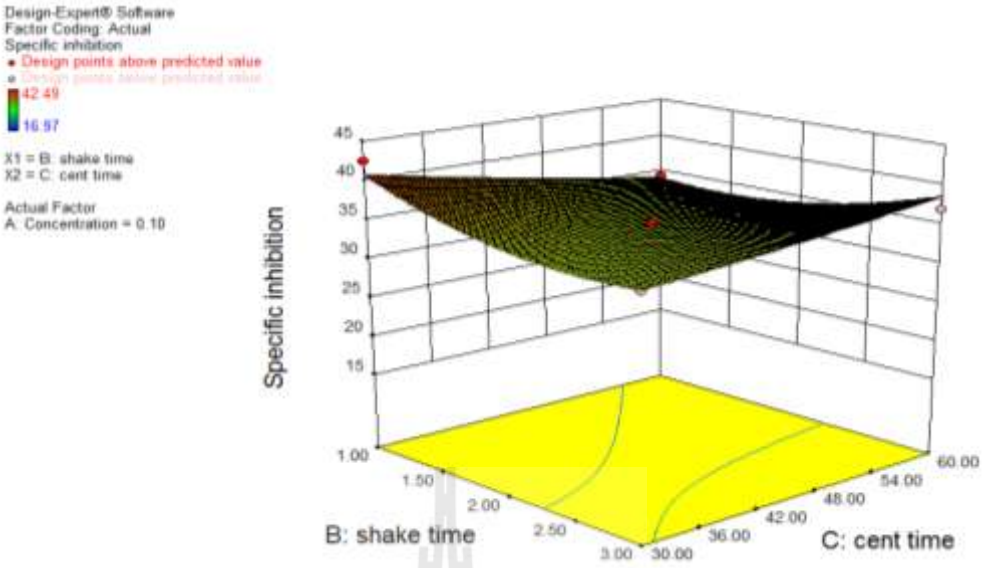

Fig. 4.2 Response surface plots indicating the interaction effect of centrifugation time and shaking time on specific inhibition activity (per mg protein)

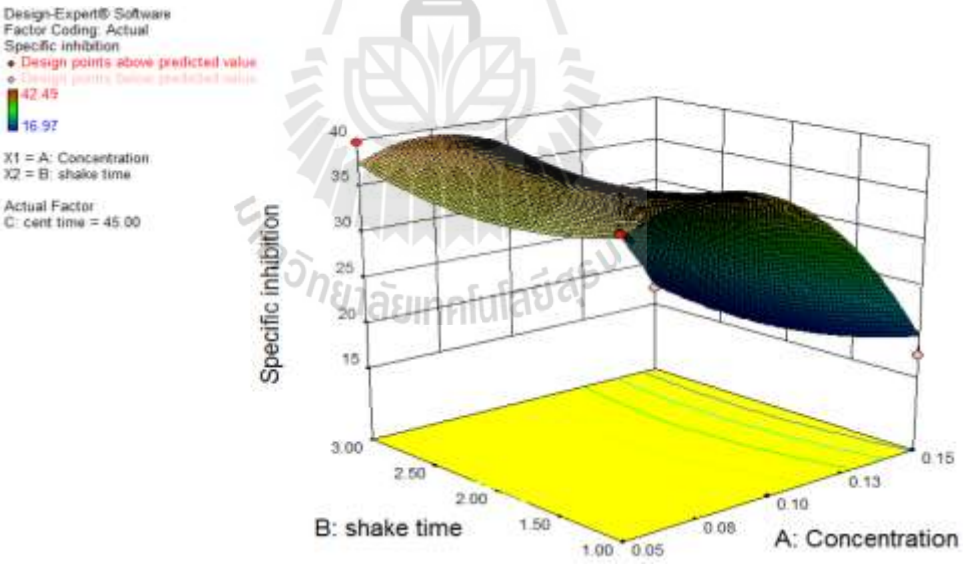

Fig. 4.3 Response surface plots indicating the interaction effect of shaking time and dried white bean powder concentration on specific inhibition activity(per mg protein)

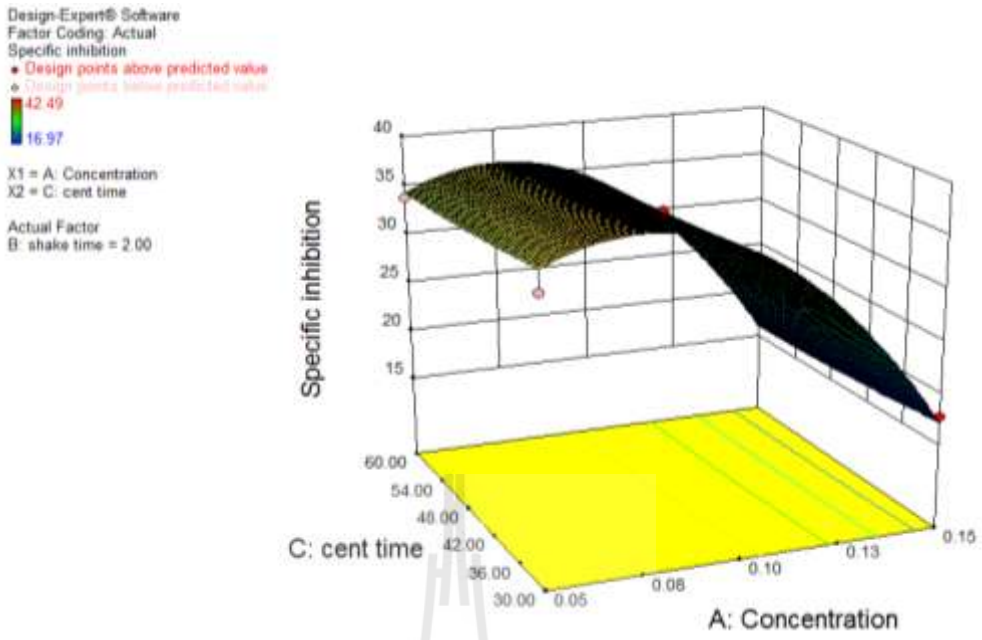

Fig. 4.4 Response surface plots indicating the interaction effect of centrifugation time and dried white bean powder concentration on specific inhibition activity (per mg protein)

From response surface methodology get optimize condition of extraction is concentration of PBS at 0.08 M , extraction time 1.5 hour and separation time 30 min.

The resultant model obtained was expressed by Equation below. The R-Squared and adjusted R-Squared values obtained of 0.9767 and 0.9707, respectively indicated that the model obtained, explained over 99% of specific inhibition activity variation.

Specific inhibition = 40.76875+573.83846 * Concentration

-14.99933 * shake time-0.39292* cent time

-15.80000 * Concentration * shake time

+0.17183* shake time * cent time

-3638.69231 * Concentration^2+2.12077* shake time^2

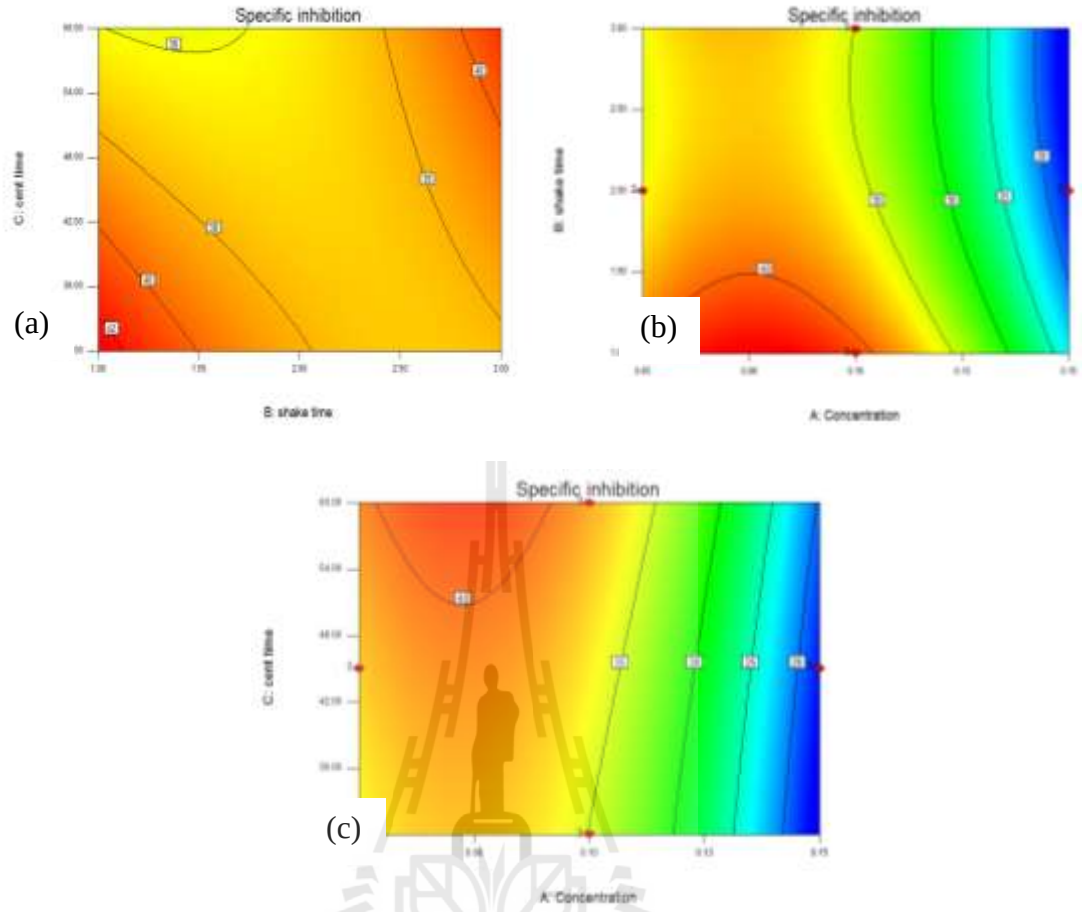

Fig. 4.5 Contours showing influence of (a) separation time- extraction time, (b) concentration of PBS -extraction time and (c) separation time- concentration of PBS on residual *Phaseolus vulgaris* ∝-amylase inhibitor (∝-AI) inhibitory activity

Table 4.6 Specific inhibition activity of trade white kidney bean extracts

Extract	Specific inhibition activity (per mg of protein)
Sample 1	34.31±0.66.[a]
Sample 2	34.42±1.17[a]
Sample 3	33.96±1.08[ba]
Trade 1	23.54±2.43[b]
Trade 2	10.69±0.21[c]

All values are means of triplicate determinations ± standard deviation.

Means within columns with different letter (a, b, c) are significantly different ($p < 0.05$)

.

When analyze specific inhibition activity in white kidney bean extract compared with commercial extract in the market and dietary supplement shown that white kidney bean extract have specific inhibition activity more than commercial extract.

Table 4.7 Specific inhibition activity (per mg protein) after In vitro digestion

Extract	Specific inhibition activity (per mg of protein)
Sample 1	24.31±0.66.[a]
Sample 2	23.92±0.97[a]
Sample 3	23.76±1.08[ba]
Trade 1	11.97±4.14[b]
Trade 2	5.53±1.31[c]

Sample are means of white kidney bean extract from adaptation method.

Trade are means of trade white kidney bean extract.

All values are means of triplicate determinations ± standard deviation.

Means within columns with different letter (a, b, c) are significantly different ($p < 0.05$)

After in vitro digestion, specific inhibition activity of white kidney bean extract and commercial extract decreased from before digestion because in vitro digestion have low pH and have many enzyme that can inactivate inhibition activity of extract and denature partially α-amylase inhibitor but the existence of a number of relatively large trypsin-resistant peptides in extract has also been observed similarly reported by Bradbear and Boulter, (1984).

4.4 Gel electrophoresis

Proteins belonging to the mixed αβ type were electrostatically better optimized than pure α-helical or β-strand structures. Proteins stabilized by disulfide bonds showed a lower degree of electrostatic optimization. Finally, the electrostatic interactions in a native protein were effectively optimized by rejection of the conformers that lead to repulsive charge–charge interactions. In figure 4.5 suggests that protein in white kidney bean extract from optimize method similar to commercial white kidney bean extract. This result suggested that both protein in white kidney bean extract from optimize method and commercial white kidney bean extract may be same protein that can inactivate α-amylase enzyme. This was similar to white kidney bean extract previously reported by Obiro et al., 2008 α-amylase inhibitor show that 30-35 kDa are associated with endoplasmic reticulum, while 14 and 19 kDa are associated with Golgi body and storage vacuoles. Marshall and Lauda, 1975 reported α-amylase inhibitor is a protein with molecular weight in the range 45,000 to 50,000. Wang et al., 2011 reported α-amylase inhibitor from extract contained two peptide fractions with the molecular weight ranged between 14.4 and 21.5 kDa, together with another two peptide fractions around 30 to 35 kDa. This profile was similar to the aAI profile for a white common bean variety reported by Tormo et al., (2006).

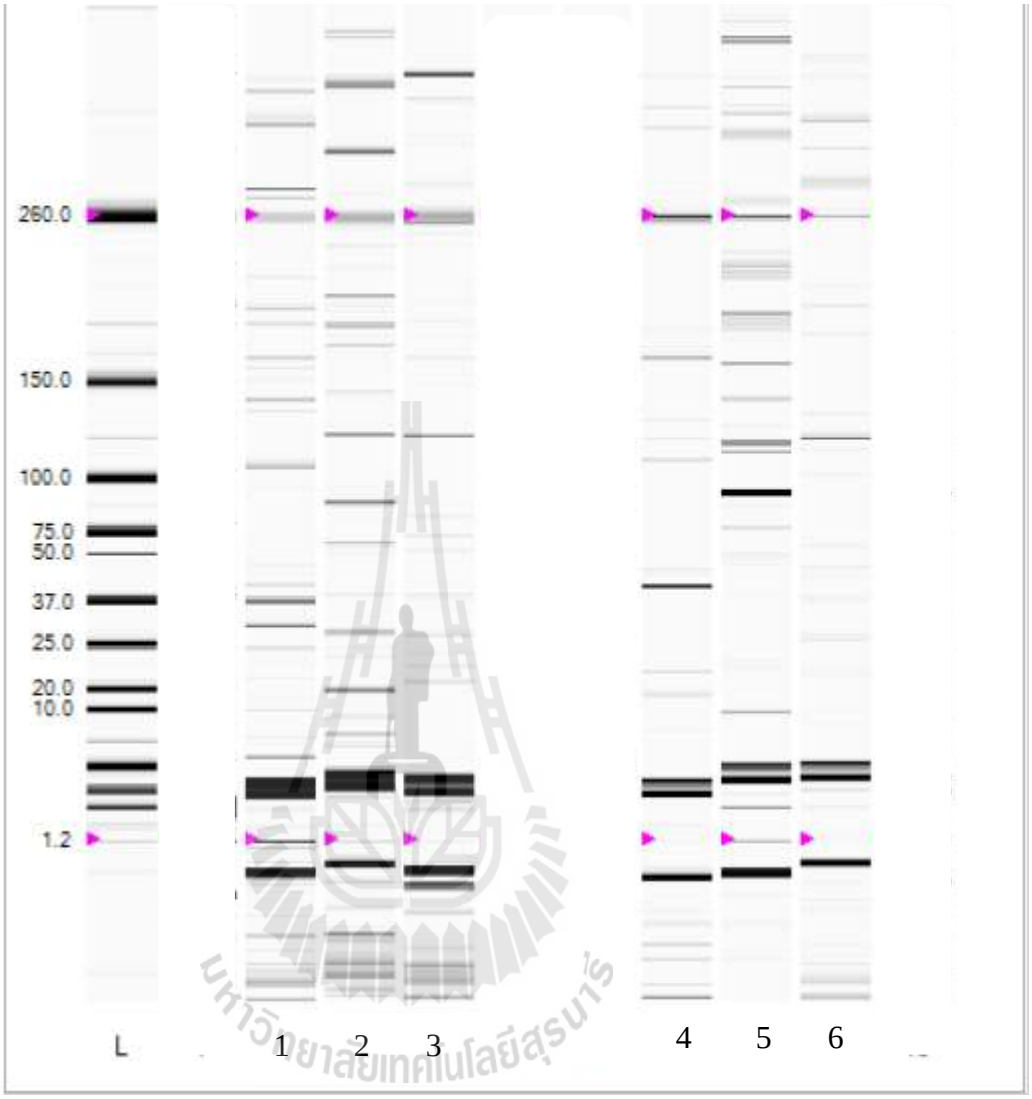

Fig 4.6 Polypeptide pattern of α-amylase inhibitors (1, 2, 3 are white kidney bean extract from adaptation method 4, 5, 6 are trade white kidney bean extract)

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APPENDICES A

How to prepare a chemical used in the analysis.

How to prepare a chemical used in the analysis

1. phosphate buffer

Stock solutions:

0.2M dibasic sodium phosphate 1 liter

$Na_2HPO_4*2H_20$	(MW = 178.05)	35.61	g
or			
$Na_2HPO_4*7H_20$	(MW = 268.07)	53.65	g
or			
$Na_2HPO_4*12H_20$	(MW = 358.14)	71.64	g
+ distilled water to make		1	liter

0.2M monobasic sodium phosphate 1 litter

$NaH_2PO_4*H_20$	(MW = 138.01)	27.6	g
or			
$NaH_2PO_4*2H_20$	(MW = 156.03)	31.21	g
+ distilled water to make		1	liter

Working buffer: 0.1M 100 ml

Mix X ml of 0.2M dibasic sodium phosphate with Y ml monobasic sodium phosphate. Dilute to 100 ml with ddH_20 or dilute 1:1 with fixative.

pH (25 C)	X ml	Y ml
5.8	4.0	46.0
6.0	6.15	43.75
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.75	4.25
8.0	47.35	2.65

Osmolarity is adjusted by varying the molarity of phosphates or by the addition of sucrose, glucose or sodium chloride.

2. NaCl 1 %

   NaCl 1 g

   Distilled water 100 g

3. 200 mM succinate buffer pH 3.8 containing 10 mM CaCl

   Succinic acid 0.2M = 23/62 g/1

   $C_4H_60_2$ (MW = 118.09)

   Add 0.2 M NaOH to desired pH. Dilute with distilled water to desired molarity.

4. 0.1 M HCl

HCl	3.65	g

Adjust the volume with distilled water to complete 1000 ml

5. Coomassie Blue solution

Coomassie brilliant blue R-250	25	g
Distilled water	100	ml
Gracial acetic acid	50	ml
Methanol	200	ml

Adjust the volume with distilled water to complete 500 ml.

6. 20 mM acetate buffer pH 4.5

Sodium acetate	0.2M = 27.2 gm/1
$CH_3CO_2Na*3H_20$	(MW - 136.09)
Acetic acid	0.2M
CH_3COOH	(MW = 60)

Add sodium acetate to acetic acid to give desired pH. Dilute with distilled water to desired molarity.

7. dinitrosalicylic acid reagent (DNS) 1%

Dinitrosalicylic acid	10	g
Phenol	2	g
Sodium sulfite	0.5	g
Sodium hydroxide	10	g

Adjust the volume with distilled water to complete 1000 ml

8. 20 mM $CaCl_2$

   $CaCl_2$ 2.22 g

   Adjust the volume with distilled water to complete 1000 ml

9. 10 mM NaCl

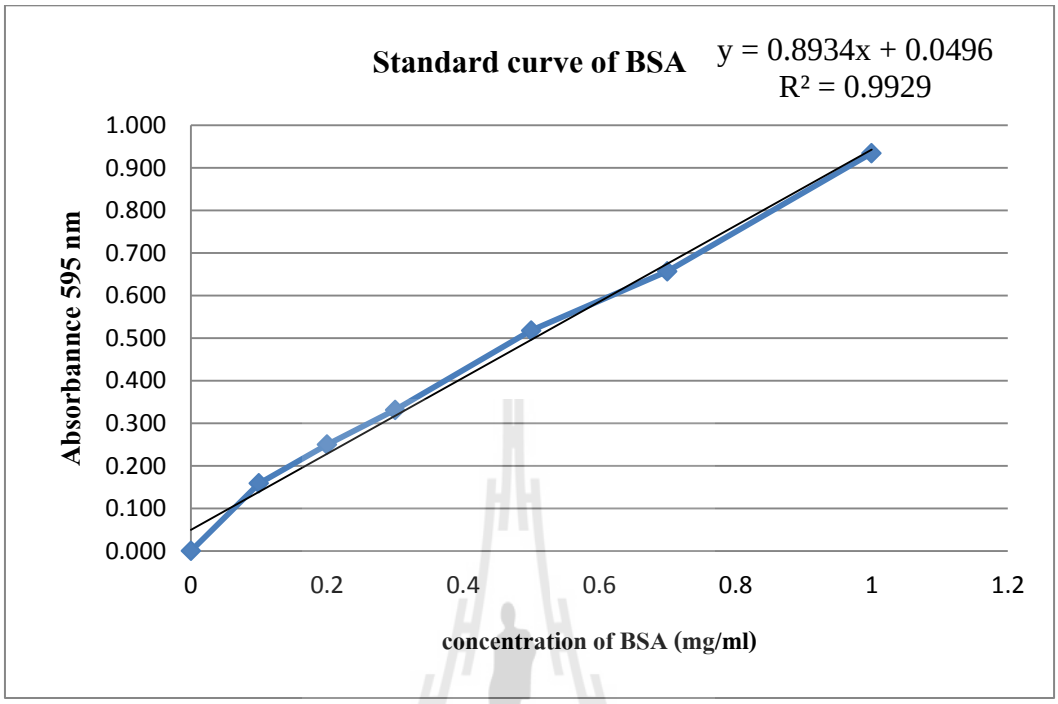
   NaCl 0.585 g

   Adjust the volume with distilled water to complete 1000 ml

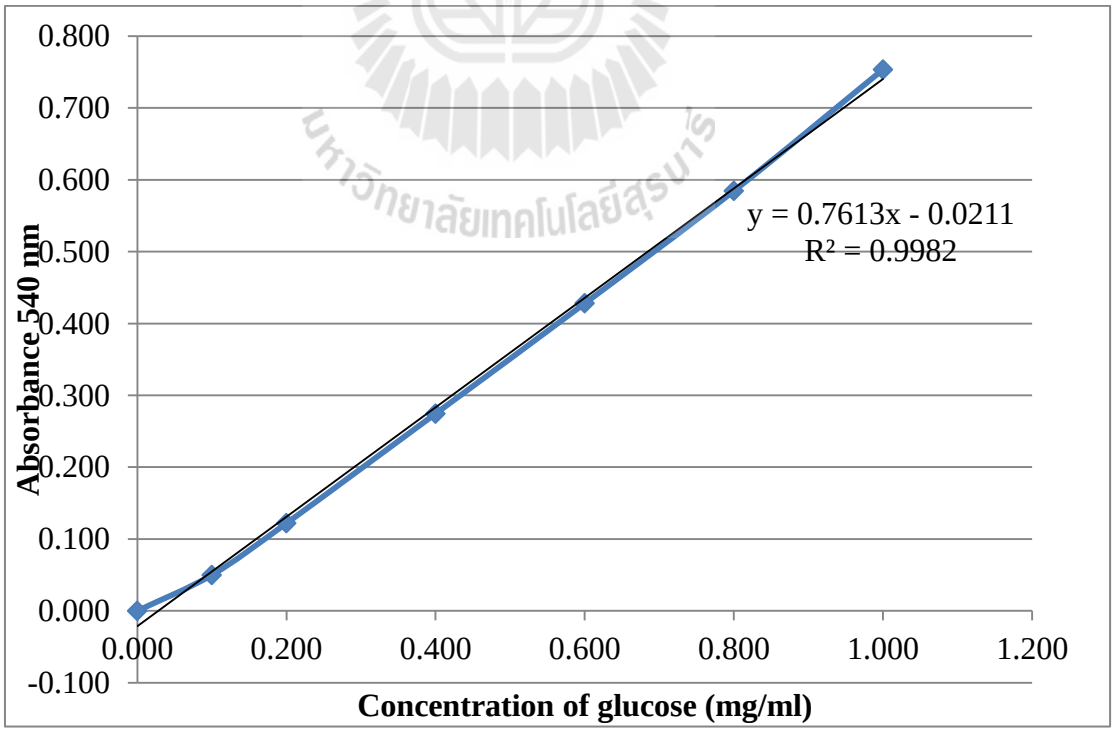
APPENDICES B

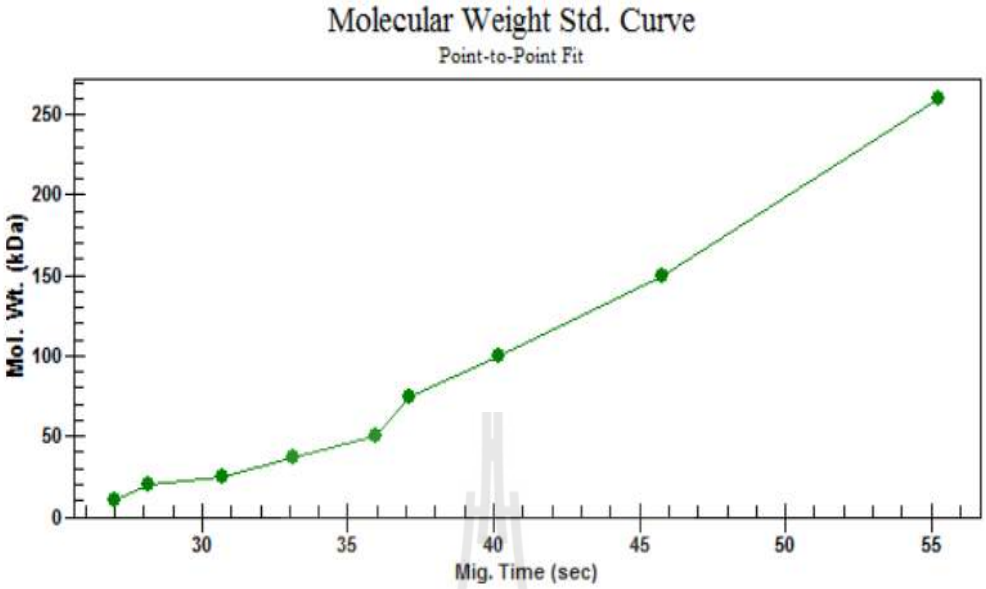
Standard curve

Standard curve of BSA



Standard curve of glucose





APPENDICES C

Statistical analysis

The One-way ANOVA for yield response.

ANOVA

Model	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	599.15	14	42.79	9399.76**	0.00
Within Groups	0.14	30	0.005		
Total	599.29	44			

**: significant at p> 0.01

The One-way ANOVA for enzyme inhibitory activity response.

ANOVA

Model	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	668.02	14	47.72	102.36**	0.00
Within Groups	13.98	30	0.47		
Total	682.00	44			

**: significant at p > 0.01

BIOGRAPHY

Jutarat Manatwiyangkool was born on January 22nd, 1988 in Bangkok Province, Thailand. She studied for high school dipoma at Wisutthikasattree School (1999-2005) in Sumutprakan Province. She received her Bachelor's Degree in Science (Food Technology) from Suranaree University of Technology in 2009. During study, she participated in the food scientist competition "FOSTAT-Nestle Quiz Bowl 2008". She started to study for her Master Degree in Food Technology at Suranaree University of Technology, Nakhon Ratchasima, Thailand. During graduate study, she was a teaching assistant in the food processing laboratory and the food engineering laboratory. The results from her research were also presented in several articles including;