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Amylase Inhibitors in Plants: Structures, Functions and Applications

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ABSTRACT

The α-amylase inhibitors have been purified and characterized from several plant species. Six classes of α-amylase inhibitors have been classified by their structures. They play important roles in the control of endogenous amylases as well as the protection against pathogens and pests. The purification methods, classification, function, inhibition mechanism together with their applications in agricultural, clinical and industrial processes have been discussed in this review.

Keywords: α-amylase inhibitor, knottin-type, Kunitz-like, cereal-type, γ-thionin-like, thaumatin-like, lectin-like
Abbreviations: α-AI, α-amylase inhibitor from kidney bean; **AMY2**, barley amylase 2**; BASI**, barley α-amylase/subtilisin inhibitor; **CM**, carboxymethyl; **DEAE**, diethyl aminoethyl; **EDI**, emmer dimeric inhibitor; **FBP**, fructose-1,6-bisphosphate; **HPLC**, high performance liquid chromatography; **kDa**, kilo Dalton; **K_i**, inhibition constant; **MS/MS**, tandem mass spectrometry; **PBE**, Polybuffer exchangers; **PEG**, polyethylene glycol; **PvCAI**, α-amylase inhibitor from *P. vulgaris*; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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INTRODUCTION

Amylase inhibitors are substances that bind to α-amylases making them inactive. They can inhibit the endogenous α-amylases, insect α-amylases and mammalian α-amylases. Amlyase inhibitors in plants act as the plant defense system against pests and pathogens. Two classes of α-amylase inhibitors have been studied so far namely non-proteinaceous and proteinaceous inhibitors. The non-proteinaceous inhibitors were found in plants and *Streptomyces*. Trestatins from *Streptomyces galbus* inhibited mammalian α-amylases (Yokose *et al*. 1983). Ascorbic acid and its derivatives are

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effective against malt, bacterial pancreatic and salivary α-amylase (Abell *et al*. 1998). The pseudo-oligosaccharides such as acarbose, isoacarbose, acarviosine-glucose (Kim *et al*. 1999), hibiscus acid from roselle tea (Hansawasdi *et al*. 2000) and cyclodextrins (Koukiekoulo *et al*. 2001) inhibit porcine and human pancreatic α-amylase. The acarbose is a strong inhibitor while the α-cyclodextrin is a weak inhibitor against the porcine pancreatic α-amylase. Both of them act as a mixed noncompetitive inhibitor (Koukiekoulo *et al*. 2001).

The proteinaceous inhibitors are also found in plants and *Streptomyces*. Haim (Murao *et al*. 1980), Paim (Murao *et al*. 1983) and Tendamistat (Vértesy *et al*. 1984) from *Streptomyces* inhibit α-amylases from mammals, *Streptomyces* sp. and *Bacillus* sp. no. 195. The plant proteinaceous α-amylase inhibitor was first reported in wheat (Chrzaszcz and Janicki 1933). The proteinaceous α-amylase inhibitors were later characterized from beans (Bowman 1945), acorns (Stankovic and Markovic 1960), mangoes (Matto and Modi 1970), rye (Granum 1978), millet (Shivaraj and Pattabiraman 1980), maize (Blanco-Labra and Iturbe-Chinas 1981), peanut (Irshad and Sharma 1981), barley (Mandy *et al*. 1983) and sorghum (Kutty and Pattabiraman 1986). Heat treatment and chromatographic method were used to purify the bean α-amylase inhibitor (Marshall and Lauda 1975; Moreno and Chrispeels 1989; Moreno *et al.* 1990) whereas the precipitation and affinity chromatography were used to purify the bifunctional α-amylase inhibitor (Kumar *et al.* 1998; Islamov and Fursov 2007; Bonavides *et al.* 2007). The α-amylase inhibitors isolated from various sources have different actions. For example, a bean α-amylase inhibitor inhibits mammalian and insect α-amylases (Ishimoto and Crispeels 1996) while a corn α-amylase inhibitor (zaematin) inhibits the insect α-amylase (Schimoler-O'Rourke *et al.* 2001). Furthermore, a bifunction inhibitor (α-amylase amylase/subtilisin inhibitor) has effective against an endogenous plant α-amylase (Yamagata *et al.* 1998) as well as animal, insect, and bacteria α-amylases (Takase 1994, Feng *et al.* 1996, Behnke *et al.* 1998). Six types of plant proteinaceous α-amylase inhibitors have been reported. These inhibitors are used in many agricultural, clinical and industrial processes. They can be used in rye bread preparation to improve dough development characteristics (Torronen *et al.* 1992). In the medical field, the inhibitor is useful to treat diabetes mellitus (Tormo *et al.* 2006), obesity type-2 (Obiro *et al.* 2008) and cardiovascular disease (Chiasson *et al.* 2003; Delorme and Chiasson 2005). The inhibitor can also be used to control the rate of starch degradation in brewing industry (Jones *et al.* 1997).

CLASSIFICATION OF PROTEINACEOUS α-AMYLASE INHIBITORS IN PLANTS

The plant α-amylase inhibitors are abundant in cereals and Leguminosae. These inhibitors exhibit specificities against different α-amylases. Some of them act against only mammalian α-amylases or insect α-amylases while others have high affinity for both mammalian and insect α-amylases. They were grouped into six classes by Richardson (1990) based on their similarity in sequences and three dimensional structures; knottin-type, Kunitz-like, cereal-type, γ-thionin-like, thaumatin-like and lectin-like.

Knottin-type α-amylase inhibitors

The knottin-type α-amylase inhibitor is the smallest proteinaceous α-amylase inhibitor. The α-amylase inhibitor from Amaranth seed (*Amaranthus hypochondriacus*) specifically inhibits the α-amylase from *T. molitor*, *Tribolium castaneum*, *Hypothenemus humpei* and *Prostephanus truncatus* (Sánchez-Hernández *et al*. 2004). The inhibitor activity was increased in insect-damaged leaves. Moreover, exogenous methyl jasmonate or abscisic acid treatment was also induced the α-amylase inhibitor activity accumulation. The amaranth α-amylase inhibitor consists of 32 amino acid residues with three disulfide bonds (Chagolla-Lopez *et al.* 1994). The structure of this inhibitor contains a knottin fold which is three antiparallel β strands and a characteristic disulfide topology (**Table 1**) (Lu *et al.* 1999; Martins *et al*. 2001).

Kunitz-like α-amylase inhibitors

The Kunitz-like α-amylase inhibitor contains 180 amino acid residues with four cysteines. They are present in cereals such as barley, wheat and rice. The barley α-amylase/subtilisin inhibitor is a β-trefoil fold protein related to a soybean trypsin inhibitor that inhibits α-amylase isozyme 2 which is synthesized in the barley seed during germination. This inhibitor is a double function inhibitor which also inhibits a serine protease of subtilisin family (Mundy *et al.* 1983). It has a molecular weight of 19,879 Da, as determined by mass spectrometry. It inhibits α-amylase isozyme 2 catalyzed starch hydrolysis with K_i of 0.09 nM (Bønsager *et al.* 2003). Amino acid sequences of the barley α-amylase/subtilisin inhibitor exhibit homology to the wheat α-amylase inhibitor and the rice α-amylase inhibitor at 92 and 58%, respectively (Franco *et al.* 2004). The crystal structure of enzyme-inhibitor complex suggested that the barley α-amylase/subtilisin inhibitor binds to A and B domains of α-amylase isozyme 2 (**Table 2**) (Vallée *et al.* 1998).

Rice α-amylase/subtilisin inhibitor has a molecular weight of 21 kDa. It inhibits subtilisin and α-amylase from *Tribolium castaneum* (Ohtsubo and Richardson 1992). The expression of rice α-amylase/subtilisin inhibitor occurred in the outermost part of rice grain and the subcellular site of aleurone cell during the late milky stage in developing seeds (Yamagata *et al.* 1998; Lin *et al.* 2006). The inhibitor weakly inhibits a germinated seed α-amylase but it inhibits a rice α-amylase stronger than a barley α-amylase (Yamagata *et al.* 1998).

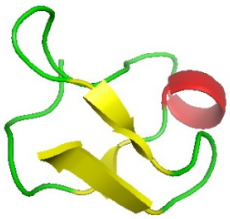
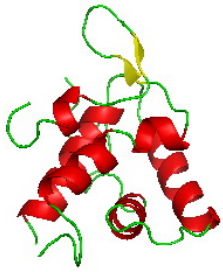
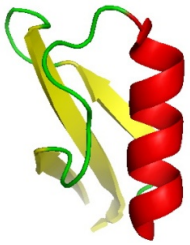
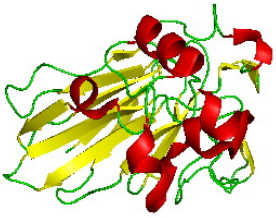
The Kunitz-like α-amylase inhibitor is also found in legume such as *Delonix regia*. The inhibitory activity of a legume α-amylase inhibitor differs from a cereal α-amylase inhibitor. The rice α-amylase/subtilisin inhibitor inhibits a endogenous and insect α-amylases as well as subtilisin (Mundy *et al.* 1983, 1984; Yamagata *et al.* 1998) whereas the legume α-amylase inhibitor inhibits mammalian and insect α-amylases (Alves *et al.* 2009). Furthermore, *D. regia* synthesizes a multiple family of Kunitz-like α-amylase inhibitors with molecular weights of 6, 20 and 24 kDa. Bioassay suggested that the inhibitor reduced larval development and increased mortality of *Callosobruchus maculatus* and *Anthonomus grandis* (Alves *et al.* 2009).

Cereal-type α-amylase inhibitors

The cereal-type α-amylase inhibitors contain 120-160 amino acid residues forming five disulfide bonds. They are present in wheat, barley, rye and ragi (Indian finger millet). These inhibitors are bifunctional α-amylase and trypsin inhibitors that inhibit mammalian, insect and bacterial α-amylases. The cereal-type α-amylase inhibitors differ from the Kunitz-like α-amylase inhibitors in their tertiary structures. The cereal-type α-amylase inhibitors contain five α-helices arranged in up-and-down manner and all cysteine residues form disulfide bond (**Table 1**) (Oda *et al.* 1997) whereas the Kunitz-like α-amylase inhibitor is a β-trefoil fold protein.

Two forms of wheat inhibitors have been described, inhibitor 0.19 and 0.28, which differ in their gel electrophoretic mobility relative to bromophenol blue (Silano *et al.* 1973, 1975). The inhibitor 0.19 is a dimer with a molecular weight of 24 kDa (Silano *et al.* 1975). It inhibits α-amylase from human saliva (Feng *et al.* 1996), pig pancreas (Fransco *et al.* 2000), chick pancreas (Buonocore *et al.* 1984), *T. molitor* (Buonocore *et al.* 1980), *Sitophilus oryzae* (Feng *et al.* 1996) and *Bacillus subtilis* (Takase 1994). The inhibitor 0.19 caused growth inhibition of *T. castaneum* larvae and a substantial loss of weight in adult insects (Feng *et al*. 1996). The inhibitor 0.28 is a monomer with a molecular weight of

Table 1 Structures of α-amylase inhibitors.

Inhibitor	Source	α-Amylase target	Determined type	Example of PDB code	3D structure
knottin-type	amaranth	*Tenebrio molitor*	NMR spectroscopy (Lu *et al.* 1999; Martins *et al.* 2001)	1QFD 1HTX	
Kunitz-like	barley	Barley α-amylase isozyme 2	n.d.		
cereal-type	wheat, barley, rye and Indian finger millet	porcine pancreatic *Tenebrio molitor*	Crystallography (Oda *et al.* 1997; Gourinath *et al.* 2000) NMR spectroscopy (Strobl *et al.* 1995)	1HSS 1B1U 1BIP	
γ-thionin-like	sorghum	*Locusta migratoria* *Periplaneta Americana*	NMR spectroscopy (Bruix *et al.* 1993)	1GPT	
thaumatin-like	maize	porcine pancreatic *Tribolium castaneum* *Sitophilus zeamis* *Rhizoperta dominica*	Crystallography (Batalia *et al.* 1996)	1DU5	
lectin-like	beans	mammalian *Callosobruchus maculatus* *Callosobruchus chinensis* *Tenebrio molitor* *Zabrotes subfasciatus*	n.d.		

12 kDa (Silano *et al.* 1975). It is highly active against the α-amylase from *T. molitor*, *Tribolium castaneum*, *Sitophilus oryzae* (Silano *et al.* 1975; Feng *et al.* 1996) and weakly inhibits mammalian and avian α-amylases (Silano *et al.* 1975). Kinetic studies revealed that the molar combining ratio for the α-amylase-0.19 inhibitor and α-amylase-0.28 inhibitor complexes are 1:1 and 1:2, respectively (Buonocore *et al.* 1980; Oneda *et al.* 2004). The wheat α-amylase inhibitor inhibits the porcine pancreatic α-amylase in a competitive manner when a *p*-nitrophenyl-α-D-maltoside is used as a substrate (Oneda *et al.* 2004). By using starch as substrate, the mode of inhibition is noncompetitive (Islamov and Fursov 2007).

The bifunctional α-amylase/trypsin inhibitor from ragi can inhibit both porcine pancreatic amylase and *T. molitor* α-amylases. It is a stable monomer of 122 amino acids with five disulfide bonds. The globular inhibitor consists of four α-helices in a simple up-and-down topology and a small antiparallel β-sheet (Strobl *et al.* 1995; Gourinath *et al.* 2000). The trypsin binding side is a canonical substrate-like conformation region while α-amylase binding site is located at the N-terminal region (Maskos *et al.* 1996; Alam *et al.* 2001). The ragi α-amylase/trypsin bifunctional inhibitor exhibited purely competitive inhibition of the porcine pancreatic α-amylase when *p*-nitrophenylmaltoside is used as a substrate. By using *p*-nitrophenylmaltoheptaoside and starch as a substrate, the mode of inhibition is complex (Maskos *et al.* 1996; Alam *et al.* 2001).

The cereal-type α-amylase inhibitor was also found in a maize seed. The corn bifunctional inhibitor is a 155 amino acid precursor protein including 28 amino acid signal peptide (Wen *et al.* 1992). The inhibitor has a molecular weight of 13.6 kDa and can inhibit the human trypsin as well as the insect α-amylase (Behnke *et al.* 1998).

A dimeric inhibitor which inhibits the human salivary α-amylase was purified from an emmer (*Triticum dicoccon* Schrank) seed. The purified protein was analyzed by MS/MS after the separation on two-dimensional gel electrophoresis. The MS/MS data indicated that the inhibitor was composed of 2 proteins, emmer dimeric inhibitor 1 (EDI-1) and emmer dimeric inhibitor 2 (EDI-2). Amino acid sequence of EDI-1 and EDI-2 share 89-98% sequence identity with related protein from *Triticum aestivum*, 0.19 and 0.53 inhibitor (Fontanini *et al.* 2007).

γ-thionin-like α-amylase inhibitors

A 5 kDa γ-thionin-like α-amylase inhibitors contain 47-48 amino acid residues, sulfur rich and form part of the γ-thionin-like superfamily. Members of this superfamily are involved in plant defense through a remarkable variety of mechanisms: modification of membrane permeability, inhibition of protein synthesis (Bloch and Richardson 1991; Méndez *et al.* 1996) and proteinase inhibition (Wijaya *et al.* 2000). The inhibitors isolated from different plants show different and unique activity. Sorghum and papaya γ-thionin inhibit the insect α-amylase (Bloch and Richardson 1991; Nitti *et al.* 1995; Farias *et al.* 2007) whereas cowpea γ-thionin and γ-thionin like soybean have effective against the bacterial amylase (Franco *et al*. 2006; Choi *et al.* 2008). A three dimension structure of barley and wheat γ-thionins has been solved by NMR. The γ-thionin consists of triple stranded antiparallel β-sheets, an α-helix and the corresponding connecting loop (Bruix *et al*. 1993) (**Table 1**).

Thaumatin-like α-amylase inhibitors

Zeamatin and α-amylase/trypsin inhibitor are 22 kDa proteins isolated from maize and barley, respectively. These proteins are homologous to the sweet protein thaumatin and pathogenesis-related proteins (Lázaro *et al*. 1988; Schimoler-O'Rourke *et al.* 2001). The expression of thaumatin was induced by pathogen attracted and abiotic stresses such as mercuric chloride, high salt, high temperature and UV light (Frendo *et al.* 1992; Malehorn *et al.* 1994). The thaumatin-like α-amylase inhibitors contain 173-235 amino acid residues forming 5-8 disulfide bonds. They inhibit the porcine pancreatic trypsin, insect α-amylase (Schimoler-O'Rourke *et al.* 2001) and microbial α-amylase (Malehorn *et al.* 1994).

The zeamatin was purified from maize using ammonium sulfate precipitation cation exchange chromatography and hydrophobic chromatography. It has a molecular weight of 22 kDa and can inhibit the porcine pancreatic trypsin and the insect α-amylases, such as *Tribolium castaneum*, *Sitophilus zeamis* and *Rhizoperta dominica* (Schimoler-O'Rourke *et al.* 2001). It also inhibits growth of *Candida albicans*, *Neurospora crassa* and *Trichoderma reesei* (Robert *et al.* 1990). The maize zeamatin has 227 amino acids including 21 residues signal peptide (Malehorn *et al.* 1994). It is a dimeric protein that consists of 13 β-strands, 11 of 13 β-strands form β-sandwich at the core of the protein (**Table 1**) (Batalia *et al.* 1996).

Lectin-like α-amylase inhibitors

Lectin-like α-amylase inhibitors contain 240-250 amino acid residues forming five disulfide bonds. The inhibitors are present in leguminosae seed, such as common bean, scarlet runner bean and cowpea. The common bean α-amylase inhibitor can be grouped by their primary structures and their inhibitory activities against weevil into three isoforms called αAI-1, αAI-2 and αAI-3. The αAI-1 inhibits the mammalian α-amylase as well as the α-amylases in the midgut of *C. maculatus* and *C. chinensis* whereas the αAI-2 inhibits the α-amylase in the midgut of *Z. subfasciatus* (Ishimoto and Crispeels 1996). The study of interaction between α-AI2 and *Z. subfasciatus* α-amylase suggested that the formation of enzyme-inhibitor complex is time dependent and occurs maximally at pH 5.0 or below (Grossi de Sá *et al.* 1997). The α-AI1 and α-AI2 share 78% sequence identity which differ in an important region that is part of the site where the enzyme binds to the inhibitor.

In addition to common bean, the α-amylase inhibitors are distributed in some other legumes of the genus *Phaseolus*, such as *P. coccineus* (scarlet runner bean), *Phaseolus polyanthus* and *Phaseolus acutifolius* (tepary bean) (Blanco-Labra 1996; Pueyo and Delgado-Salinas 1997). The inhibitory activity of the α-amylase inhibitor from scarlet runner bean and *P. polyanthus* are similar to the α-AI1 from common bean (Pueyo and Delgado-Salinas 1997). Tepary bean seed has two isoforms of the α-amylase inhibitors, designnated αAI-Pa1 and αAI-Pa2. Both isoforms exhibit broad specificity toward α-amylases. The αAI-Pa1 inhibits the larval α-amylases of *C. chinensis and C. maculatus* but not that of *Z. subfasciatus*, whereas the αAI-Pa2 inhibits the larval α-amylase of these three bruchids. Both isoforms also inhibit the larval amylase of cereal storage pest, including the yellow mealworm (*Tenebrio molitor*) and confused flour beetle (*Tribolium confusum*), but they do not inhibit the mammalian α-amylases (Yamada *et al*. 2001). Like amylase inhibitor from the red kidney bean, the active from of the αAI-Pa2 is a heterodimer, whereas the αAI-Pa1 is consisted of only a single glycopolypeptide (Yamada *et al.* 2005).

α-amylase inhibitors have also been identified in other legumes of genus *Vigna*, such as *Vigna sublobala*, cowpea (*Vigna unguiculata*) (Piergiovanni and Gatta 1994) and mungbean (*Vigna radiata*) (Haq *et al.* 2005). Kokiladevi *et al.* (2005) compared inhibitory activity of α-amylase inhibitors in the seed of *Vigna* genotypes and found that *Vigna umbellata*, *V. sublobata* and *Vigna glabracens* belonged to a group with higher levels of inhibitory activity. *Vigna trilobata* showed a moderate level of inhibition while *V. radiata* and *V. unguiculata* exhibited a lower inhibitory activity.

FUNCTION OF α-AMYLASE INHIBITORS IN PLANTS

Two roles of α-amylase inhibitors have been identified. The primary function of inhibitors is protecting the seed against microorganisms and pests. Protein extracts from resistant and transgenic plants are effective against α-amylases from insects (Sivakumar *et al.* 2006; Farias *et al.* 2007; Barbosa *et al.* 2010; Dias *et al.* 2010), bacteria and fungi (Yamasaki *et al.* 2006). Expression of the α-amylase inhibitor genes in transgenic seeds, such as pea (Shade *et al.* 1994; Schroeder *et al.* 1995), azuki bean (Ishimoto *et al.* 1996), chickpea (Sarmah *et al.* 2004; Ignacimuthu and Prakash 2006), rye (Dias *et al.* 2005), cowpea (Solleti *et al.* 2008) and tobacco (Dias *et al.* 2010), inhibit growth and development of insect pests. Moreover the inhibitors also reduce fecundity, number of adult emerged and survival rate of bruchid weevils. Experiment under field condition revealed that transgenic peas containing the αAI-1 were resistant to damage by the pea weevil (Morton *et al.* 2000).

The other function of inhibitor is the inhibition of the endogenous α-amylase (Henry *et al.* 1992). An α-amylase inhibitor is synthesized as a seed protein during endosperm development (Jarrett *et al.* 1997). It functions as an active mediator of amylase activity in developing and germinating seeds (Mundy 1984). During germination, the α-amylase hydrolyzes endospermic starch to sugar. The bifunction cereal α-amylase/subtilisin inhibitors prevent precocious germination by inhibiting endogenous enzyme (Yamagata *et al.* 1998).

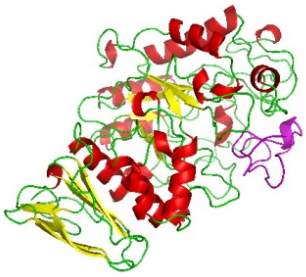
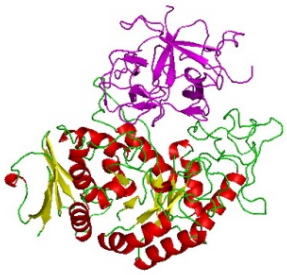
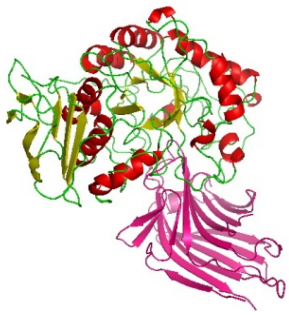
STRUCTURAL BASIS FOR THE INHIBITION MECHANISMS

The three dimensional structures of enzyme-inhibitor complex are available for inhibitors from amaranth, ragi, barley and bean. This α-amylase inhibitor interacts with residues from domain A and B which line the substrate binding site of enzyme and makes it lose activity. The bindings between inhibitors and active site of enzymes can be either direct hydrogen bond or through hydrogen bond via a water network or a water molecule (Svensson *et al.* 2004).

Structure of amaranth α-amylase inhibitor

The structure of the amaranth α-amylase inhibitor in complex with *T. molitor* α-amylase was determined by crystallography. Two segments of the inhibitor bind to the active site of *T. molitor* α-amylase blocking the central four sugar-

Table 2 α-amylase/inhibitor complex structure.

Inhibitor	Determined type	Enzyme-inhibitor complex	PDB code	3D structure[a]
knottin-type	crystallography	*Tenebrio molitor* α-amylase/amaranth α-amylase inhibitor (Pereira *et al.* 1999)	1CLV	
kunitz-like	crystallography	barley α-amylase (AMY2)/barley α-amylase/subtilisin inhibitor (Vallée *et al.* 1998)	1AVA	
cereal-type	crystallography	*T. molitor* α-amylase/ragi bifunctional inhibitor (Strobl *et al.* 1998)	1TMQ	
lectin-like	crystallography	*Tenebrio molitor* α-amylase/α-AI1 (Bompard-Gilles *et al.* 1996) porcine pancreatic α-amylase/α-AI1 (Nahoum *et al.* 1999)	1VIW 1DHK	

[a] Ribbon diagram of the complex; strand and helix are shown in yellow and red, respectively. Domain B and inhibitor are shown in green and magenta, respectively

binding subsites. In the enzyme-inhibitor complex, the residue D287 at the catalytic site of *T. molitor* α-amylase forms a salt bridge directly with R7 of the amaranth α-amylase inhibitor. The other catalytic residues, D185 and E222, are connected to the R7 side chain via an intricate water-mediated hydrogen-bond network (**Table 2**) (Pereira *et al.* 1999).

Structure of barley α-amylase/subtilisin inhibitor

The structure of β-trefoil fold protein has been solved in complex with barley amylase 2 (AMY2). The barley α-amylase/subtilisin inhibitor (BASI) binds to AMY2 with a K_i of 0.22 nM (Abe *et al.* 1993). The interaction between BASI and AMY2 is increase with decreasing pH or increasing an ionic strength (Torronen *et al.* 1992). The BASI does not directly interact with the catalytic residues (D179, E204 and D289). The enzyme-inhibitor complex contains a fully hydrated Ca^{2+} (Ca^{503}) at the central of protein interface. This ion binds water molecules with the surrounding catalytic site residues and inhibitor side chains, T170 and E168, respectively (**Table 2**) (Vallée *et al.* 1998). The mutation on BASI revealed the role of charge interaction between BASI and AMY2 because the mutant K140L D150N E168T and Y170F lost inhibitory activity. The binding between mutant Y170F decreases with increased calcium concentration suggests that the calcium ion and its solvation sphere are integral component of amylase-inhibitor complex (Bønsager *et al.* 2005).

Structure of bifunctional α-amylase/trypsin inhibitor

The structure of ragi bifunctional inhibitor has been solved in complex with *T. molitor* α- amylase. The α-amylase and inhibitor complex reveals that the N-terminal residues S1-A11 and residues P52-C55 protrude like an arrow head into the substrate-binding groove and directly target the catalytic residues. Although residues 1-5 are flexible in the solution structure of free inhibitor, they adopt a 3_{10}-helical conformation in the complex with *T. molitor* α-amylase and fill

the saccharide-binding subsites. S1 makes several hydrogen bonds with D185 and E222, while V2 and S5 from the inhibitor interact with D287 (**Table 2**) (Strobl *et al.* 1998).

Structure of bean α-amylase inhibitors

1. Interaction of the α-AI1 with mammalian α-amylases

The interaction of the α-AI1 with porcine pancreatic α-amylase and human pancreatic α-amylase occurs at the α-amylase catalytic site and the inhibition process is very similar for both enzymes. In the α-AI1-porcine pancreatic α-amylase and the α-AI1-human pancreatic α-amylase complexes, two hairpin loops of the α-AI1 form extensive hydrogen bonds and water bridged contacts with amino acid residues at the enzyme catalytic site. At the substrate binding site, two tyrosine residues (Y37 and Y186) from the two hairpin loops of the α-AI1 combine with amino acid residues (D197 and E233, respectively) at the catalytic site of the enzyme (Bompard-Gilles *et al.* 1996; Nahoum *et al.* 2000). The interactions occurring in region of subsite -1, +1 and +2 are highly conserved in the complex between inhibitor and pancreatic α-amylase. The hydrophobic interactions also occur between substrate and hydrophobic residues lining the entrance of the cleft (subsite-2 and -3). These interactions are followed by protein-protein interactions involving areas further away from the catalytic center, namely the "flexible loop" (residues 303-312), the loops comprising residues 237-240 and 347-357, and the loop formed by residues 140-150 from domain B (**Table 2**) (Nahoum *et al.* 2000; Payan 2004).

When the α-AI1 binds to the mammalian α-amylase, the flexible loop moves out towards the solvent and is pushed away by an inhibitory tight binding process. The structure of free human pancreatic α-amylase and human pancreatic α-amylase/α-AI1 complex showed that the side chain of D300 adopts clearly different positions. These indicated that, in the human pancreatic α-amylase, this residue is able to undergo conformation change (Nahoum *et al.* 2000).

The study on the interaction between the porcine pancreatic α-amylase and the α-AI1 from *P. vulgaris* suggested that the inhibition occurs after preincubation of the porcine pancreatic α-amylase and the α-AI1 before the substrate is added. When the α-AI1 binds to the porcine pancreatic α-amylase, the conformation of the porcine pancreatic α-amylase is changed (Santimone *et al.* 2004).

2. Interaction of α-amylase inhibitor with insect α-amylase

The interaction between the *T. molitor* α-amylase and the α-AI1 is similar to that in the porcine pancreatic α-amylase-α-AI1 complex, with large deviation occurring in the loop and particularly in the loop segments next to the catalytic site region. However, the structure analysis of the *T. molitor* α-amylase-α-AI1 complex indicated the strong contacts occurring between the inhibitor and the catalytic site of the porcine pancreatic α-amylase. Amino acid sequences of the human pancreatic α-amylase and the *T. molitor* α-amylase are highly conserved. Two hairpin loops of α-AI1 (Y37 and Y186) bind to D185 and E222 at the enzyme catalytic site (Nahoum *et al.* 1999).

PURIFICATION OF α-AMYLASE INHIBITORS

Different techniques have been developed for isolation and purification of proteins based on their properties, such as solubility, non-specific adsorption-desorption processes on inorganic supports, electrical charges as a function of pH and molecular size. The dependence of solubility of protein on solvent composition is very often used at the starting point for the purification processes. Salting out techniques, precipitation with organic solvents, precipitation with organic polymers, and isoelectric precipitation constitute good examples of this principle. Non-specific adsorption of proteins on certain inorganic materials, such as calcium phosphate (hydroxyapatite) or alumina gels and diatomaceous earths, followed by subsequent protein desorption have also been widely applied as the purification procedures (Harris and Angal 1990). The most commonly used technique in final step of purifications is a combining of ion-exchange and gel filtration chromatography.

Heat treatment method

Most proteins are stable at a low temperature. Above 40°C, most proteins denature and precipitate. Most of α-amylase inhibitors are heat stable proteins and can use this property for protein purification. Thermotolerant proteins were purified by incubating the cell extract at 5-10°C below their temperature stability for 15-30 min, and then removing the precipitated protein by centrifugation. The heat treatment is always used as the first step of purification to precipitate most of proteins from the crude.

Phaseolamin, an α-amylase inhibitor from kidney bean (*Phaseolus vulgaris*) was purified in a four step procedures involving heat treatment and chromatography on DEAE cellulose, Sephadex G-100 and CM cellulose. The purified protein gave a yield of 37%, with a purification of up to 32-fold over the crude extract. Phaseolamin inhibited the animal α-amylases but did not inhibit the α-amylase from plants, bacteria and fungi (Marshall and Lauda 1975). The α-amylase inhibitor from common bean seed was purified using a selective heat treatment in an acidic medium followed by an affinity chromatography using the procine pancreatic amylase coupled to an agarose bead. The purified proteins gave five different isoforms ranging from 14 to 19 kDa (Moreno and Chrispeels 1989).

The common bean, *Phaseolus vulgaris*, contains a glycoprotein that inhibits the activity of the mammalian and insect α-amylases, but not the plant α-amylases. The inhibitor was purified by heating at 70°C for 10 min. The protein precipitant was removed by centrifugation and the clear supernatant was applied to an affinity chromatography using the porcine pancreatic α-amylase coupled to a CNBr-activated Sepharose 4B. The purified inhibitor was resolved by SDS-PAGE into five bands in the range of 15 to 19 kDa. It inhibits the porcine pancreatic α-amylase but not of the barley α-amylase (Moreno *et al.* 1990). Ho and Whitaker (1993) achieved an 18.5-fold increase in the specific activity of a white kidney bean α-amylase inhibitor from the aqueous crude extract by heat treatment, ethanol fractionation, and subsequent chromatographic separation on a DEAE cellulose column.

Affinity precipitation

Affinity precipitation is a bioseparation technique that exploits the specific interaction between a target protein and a ligand coupled to a water-soluble polymer. The polymer-ligand-protein complex is precipitated by altering the conditions to change the solubility of the polymer. Changes in pH, temperature, and ionic strength as well as the addition of metal ions, water-soluble organic solvents, or oppositely charged electrolytes may be used to precipitate the polymer. The target protein can then be dissociated using methods similar to those used to elute proteins from columns in affinity chromatography (Culter 1996).

The wheat α-amylase inhibitor has been purified 4 folds with total recovery of 80% using copolymer of 1-1-vinylimidazole and *N*-isopropylacrylamide. This polymer was charged with Cu(II) and exhibited specific interaction of the metal ions to the α-amylase inhibitors. The precipitation was induced by salt and the recovery of the amylase inhibitor was achieved by dissolving the inhibitor-polymer complex in imidazole buffer and subsequent precipitation of the polymer (Kumar *et al.* 1998).

Two forms of regi α-amylase inhibitor was separated using Cu(II) loaded thermosensitive metal chelate copoly-

mer of N-isopropylacrylamide and 1-vinyl imidazole. An α-amylase inhibitor I-1 binds to the copolymer whereas the α-amylase inhibitor I-2 was recovered in the supernatant. The α-amylase inhibitor I-1 was purified 13-fold purification with 84% yield. The protein has a molecular weight of 14 kDa. It inhibits the porcine pancreatic α-amylase as well as the bovine trypsin. The 13 kDa α-amylase inhibitor I-2 was purified 4-fold purification with 27% yield (Kumar *et al.* 1998).

The double headed inhibitor of amylase and proteinase K was purified from wheat germ using heat treatment and Cu(II)-Streamline chelating resin. The inhibitor was obtained with 24-fold purification and 84% recovery. The purified protein gave a single band on SDS-PAGE at the estimated molecular weight of 21 kDa (Roy and Gupta 2000).

Aqueous two-phase system

An aqueous two-phase system of polyethylene glycol (PEG)/fructose-1,6-bisphosphate (FBP) trisodium salt was employed to purify an α-amylase inhibitor from wheat flour. The purified inhibitor is 3.2 fold increase with 79% recovery by using 11.7% (w/w) of PEG 2000 and 19% (w/w) of fructose-1,6-bisphosphate trisodium salt at pH 7.0 in the top phase (Chen *et al.* 2008).

Chromatographic methods

Chromatographic methods, such as gel filtration, ion exchange and affinity chromatography, are widely used for purification of α-amylase inhibitors from various sources. An α-amylase inhibitor was purified 125-fold from a crude extract of barley kernels by ammonium sulfate precipitation, ion exchange (DEAE sephacel) and gel filtration (Bio-Gel P 60) chromatography. The inhibitor was a protein with an approximate molecular weight of 20 kDa and an isoelectric point of 7.3 (Weselake *et al.* 1983).

The kidney bean (*P. vulgaris* L. cv. 'Tendergreen') α-amylase inhibitor has two isoforms, called α-AI1 and α-AI1′. These proteins were purified by heat treatment in acidic medium, ammomium sulfate precipitation, chromatofocusing (PBE 94 column) and gel filtration (Sephadex G-200) chromatography. The yield of α-AI1 and α-AI1′ is close to 0.05% and 0.02% (w/w), respectively. The two isoforms exhibit the same molecular weight of 43 kDa but differ in their pIs and the extent of N-glycosylation (Le Berre-Anton *et al.* 1997).

An α-amylase inhibitor was purified 82-fold from crude extract of white kidney bean (*P. vulgaris*) by reversed phase HPLC. This inhibitor is a glycoprotein with deglycosylated molecular weight of 54.847 kDa, as determined by electrospray ionization mass spectrometry. The binding constant was 2.8 μM at 55°C. The effect of salt on inhibitory activity was also determined. Chloride ion was important for full activity while calcium ion increased the initial rate of binding. Magnesium and sulfate ions, however, did not affect the inhibitory activity (Gibbs and Alli 1998).

A 21 kDa bifunctional amylase/subtilisin inhibitor was purified from rice (*Oryza sativa* L.) by ammomium sulfate precipitation, ion exchange (CM-cellulose) and gel filtration (Sephadex G-75) chromatography. The yield of subtilisin inhibitory activity was 8% from crude extract, with 93-fold purification. The inhibitor has a pI of 9.05. It inhibits the subtilisin from *Bacillus subtilis* and weakly inhibits amylase from the germinated seed (Yamagata *et al.* 1998).

The cowpea α-amylase inhibitor was purified 51-fold with a total recovery of 8.76% using an affinity chromatography on Red Sepharose CL-6B. The inhibitor was active against the α-amylases from *Bacillus* sp., *Aspergilus oryzae*, *V. unguiculata* seeds and *C. maculatus* larvae (Melo *et al.* 1999).

The corn α-amylase inhibitor was purified 20.7-fold with a total recovery of 3.6% using the ammonium sulfate precipitation, gel filtration on Sephadex G-75, HPLC Superose HR10/30 and HPLC ion exchange chromatography. Purified inhibitor has a molecular weight of 23.8 kDa and an acidic isoelectric point of 5.4. It inhibits the mammalian α-amylase as well as the α-amylase from *Fusarium verticillioides* (Figueira *et al.* 2003).

The bifuctional inhibitor was purified from mungbean (*Phaseolus aureus* or *Vigna radiata* L.) by acetic acid precipitation, ammonium sulfate precipitation, ion exchange (DEAE-cellulose) and affinity (trypsin Sepharose) chromatography. It exhibits inhibitory activity towards the trypsin-like and chymotrypsin-like serine proteinases as well as the α-amylases (Haq *et al.* 2005).

The α-amylase inhibitor from *P. vulgaris* (PvCAI) was purified by ammonium sulfate fractionation, ion exchange chromatography using DEAE cellulose and reversed phase HPLC (Dayler *et al.* 2005). The molecular weight of PvCAI was 33.330 kDa, as determined by mass spectrometry. This inhibitor inhibits larval *Z. subfasciatus* α-amylases but not the mammalian α-amylase. An NH_2-terminal amino acid sequence of PvCAI is different from other α-amylase inhibitors and it shows high identity with a plant chitinase.

The α-amylase inhibitor of cowpea weevil digestive enzyme, PpAI, was purified from white sucupira seeds (*Pterodon pubescens*) by ammonium sulfate precipitation, chromatograpy on Red Sepharose CL-6B and reversed phase HPLC. The inhibitor was obtaining with 11.7-fold purification and recovery of 46.7%. The purified PpAI had a molecular weight of 5 kDa and had specificity toward the insect enzymes. Moreover, artificial seeds containing PpAI were able to reduce larval weight by 36% and caused 55% mortality (Silva *et al.* 2007).

The α-amylase inhibitors from baru seeds (*Dipteryx alata*) were purified by ammonium sulfate precipitation, affinity chromatography (Red-Sepharose CL-6B) and reversed phase HPLC. These proteins inhibit α-amylases from *C. maculatus* and *Anthonomus grandis* (Bonavides *et al.* 2007).

The wheat bifunction α-amylase/trypsin inhibitor was purified 300-fold using ammonium sulfate precipitation, followed by affinity chromatography on trypsin sepharose. The inhibitor has a molecular weight of 14 kDa. It inhibit salivary α-amylase and trypsin. The inhibitor retains activity at high temperature but is inactive in the presence of the SH group-reducing agents (Islamov and Fursov 2007).

The α-amylase inhibitor that effectively inhibits mammalian α-amylase was purified from white kidney bean by ethanol fractional precipitation, ion exchange (CMII cellulose) and gel filtration (Sephadex G-75) chromatography (Yang *et al.* 2008).

According to the purification techniques, heat treatment and aqueous two phases system removes partially protein resulting low purity. On the other hand, affinity chromatography of trypsin sepharose or α-amylase resin gives a good yield and purity due to their specific binding between inhibitors and enzymes. Since the α-amylase inhibitors have different properties, the inhibitory activity should be tested before employ the affinity column. In addition, the combination techniques such as heat treatment and chromatographic methods are also giving high purity proteins.

APPLICATION OF α-AMYLASE INHIBITOR

Agricultural application

As the α-amylase inhibitors inhibit the digestive amylases in the midgut of insect larvae, it can be used as an insecticidal protein to prevent the growth of insect larvae that infest the seed. This has been confirmed by rearing insects on artificial diets that contain different levels of α-amylase inhibitors (Ishimoto and Kitamura 1989; Farias *et al.* 2007) and by expressing α-amylase inhibitor in transgenic plants (Altabelia and Chrispeels 1990; Shade *et al.* 1994; Schroeder *et al.* 1995; Dias *et al.* 2005, 2010). The use of insecticidal protein has the potential to benefit agricultural crop production, the environment and the consumer through the

reduced use of chemical pesticides and insecticides.

Clinical application

The α-amylase inhibitor is also known as a starch blocker because they contain substances that prevent dietary starches from being absorbed by the body. Investigation on human and animal suggested that the α-amylase inhibitor inhibits amylase activity while ingested with dietary starch (Layer *et al.* 1985). The inhibitor delays postprandial carbohydrate digestion and absorption, and lowers plasma glucose levels without altering a pancreatic growth (Koike *et al.* 1995; Choudhury *et al.* 1996). It is used to control human non-insulin dependent diabetes mellitus and obesity (Tormo *et al*. 2006; Obiro *et al.* 2008; Barrett and Udani 2011). Furthermore, the α-amylase inhibitor decreases in the progression of intima-media thickness in the carotid arteries. It reduces the risk of cardiovascular disease and hypertension (Chiasson *et al.* 2003; Delorme and Chiasson 2005).

Industrial application

The α-amylase inhibitor may be used in industrial processes where starch degradation is avoided, for example, in baking and brewing. In baking, barley amylase/subtilisin inhibitor is used to improve the baking properties of sprouted flour (Zawastikowska *et al.* 1988; Torronen *et al.* 1992). In brewing, barley amylase/subtilisin inhibitor controls the rate of starch degradation in early stage of mashing. Since the inhibitor is denatured below normal mashing temperature, the inhibitor does not affect amylase production of fermentable sugar from starch (Munck *et al*. 1985; Jones *et al.* 1997). In addition, the amylase inhibitor has been used to improve beer foam stability due to their hydrophobic interaction between the inhibitor and iso-α-acid from hop (Iimure *et al.* 2008; Okada *et al.* 2008).

FUTURE PROSPECTS

Although the α-amylase inhibitors isolated from different plant are characterized, there are a few studies of enzyme-inhibitor complex. Understand the interactions between the α-amylases and the α-amylase inhibitors help scientists to design an effective inhibitor which specific to the insect pest. Moreover, site direct mutagenesis will be used to improve the inhibitory activity. The site effect of transgenic crop should study to ensure that it is saving for human and environment. In addition, the animal and human toxicity should be tested before apply the inhibitor to human and industry.

CONCLUSIONS

The plant α-amylase inhibitors play important roles in the control of endogenous amylase as well as protection against pathogen and pests. The α-amylase inhibitors were purified and characterized. Six classes of α-amylase inhibitor, knottin-type, Kunitz-like, cereal-type, γ-thionin-like, thaumatin-like and lectin-like, have been grouped using three dimensional structures. The crystal structure of enzyme-inhibitor complex revealed that the inhibitor interacts with residues from domain A and B which line the substrate binding site of enzyme and makes it lose activity. These inhibitors are used in agricultural, clinical and industrial processes.

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