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Solubility and Manipulation of Disulfides in Puroindoline-b : Recombinant Puroindoline-b Shows Antifungal Activity

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Solubility and Manipulation of Disulfides in Puroindoline-b

Recombinant Puroindoline-b shows antifungal activity

A Thesis Submitted to the Faculty of Graduate and Postdoctoral Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

By

Kechun Wu

Your file Votre référence
ISBN: 0-494-11458-4
Our file Notre référence
ISBN: 0-494-11458-4

ABSTRACT

Wheat (*Triticum aestivum*) kernel texture (hardness) is the most important determinant of milling and end-product quality. Recent data indicate that the only difference between soft and hard textured wheat is a single amino acid mutation in one protein, puroindoline-b (PIN-b). A rare tryptophan-rich domain in this protein consists of five tryptophan residues among a stretch of seven amino acid residues. To understand how this single mutation makes hard wheat possible, thus enabling bread making, it is crucial to have a high-resolution three-dimensional structure of this protein. The prerequisite for structural elucidation of any protein is the high-quality sample preparation.

In this thesis PIN-b from a diploid wheat (*Triticum monococcum*) was chosen as the model system because its grain is soft and it has the simplest genome of all wheats. The coding sequence of PIN-b was amplified from the diploid wheat using PIN-b specific primers. It was cloned into a protein expression vector. PIN-b was expressed as a protein behind the thioredoxin (TRX-a) tag. The TRX-a-PIN-b fusion protein was purified using nickel chelating chromatography.
The immunological identity of the fusion protein was confirmed by Western blot. The PIN-b was released from the fusion protein by enterokinase proteolysis and purified using ion exchange chromatography. After glutathione treatment to facilitate full formation of the potential five disulfide bonds, PIN-b demonstrated higher fungicidal activity when compared to the non-treated PIN-b.

ACKNOWLEDGEMENT

I would like to thank Professor Illimar Altosaar for giving me the opportunity to pursue this work. Thanks to Illimar for his patience, generosity and many lessons he taught me throughout my time in his lab.

I am grateful to Dr. Natalie Goto for her many suggestions and productive discussions. I extend special thanks to Dr. Michael McBurney and Dr. Gabriel Guillet for providing valuable guidance via my thesis advisory committee. I really appreciate the constructive comments from Dr. Maxwell Hincke as my one of my thesis examiners.

My gratitude also goes to those who have helped me through the tough hurdle of writing this thesis.

I dedicate this thesis to my mother and father.

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List of Abbreviations

1D one dimensional
2ME 2-mercaptoethanol
3D three-dimensional
AACC American association of cereal chemists
AMP antimicrobial protein/peptide
APS ammonium persulfate
ATP adenosine triphosphate
BSA bovine serum albumin
CTAB cetyltrimethylammonium bromide
DPA days post-anthesis
DSB protein disulfide isomerase
GOR glutathione oxidoreductase
GSH reduced glutathione
GSSG oxidized glutathione
GSP grain softness protein
HI hardness index
IC_{50} the concentration inhibiting 50% growth
IgG immunoglobulin
IPTG isopropyl-beta-D-thiogalactoside
LB Luria-Bertani broth
MIC minimal inhibitory concentration
M.W. molecular weight
NCBI national center for biotechnology information
NMR nuclear magnetic resonance
OD optical density
nsLTP non-specific lipid transfer protein
M.W. molecular weight
PBS phosphate buffered saline
PCR polymerase chain reaction
PIN puroindoline
PMSF phenyl methyl sulfonyl fluoride
PSI particle size index
PVP polyvinylpyrrolidone
RP-HPLC reverse phase high-performance liquid chromatography
RT recombinant
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SKCS single kernel characterization system
TEMED tetramethylethylenediamine
TRX-a thioredoxin
TRX-b thioredoxin reductase

Chapter I Introduction

1.1 Wheat production and its classification

A. One protein's role in the history of agriculture

Humans eat not the whole wheat seed, as the texture is far too hard for our teeth. It is necessary to first grind wheat grain into flour for human consumption. Soft textured wheat seeds can be ground into flour with lower energy than that required for hard textured wheat. Recent data indicate that the only difference between soft and hard textured wheat is a single amino acid mutation in one protein, puroindoline-b (PIN-b). As this point mutation made hard wheat possible, thus enabling flour grinding and bread making, it is conceivable to assume that this mutation may be one of the most important single mutations in the history of agriculture (Giroux and Morris, 1998; Morris, 2002).

B. Wheat: Humanity's Number One Food

Wheat was one of the first grain crops domesticated by humans. Its cultivation began in the Neolithic period (around 10,000 to 2,000 B.C). Bread wheat is known to have been grown in the Nile Valley around 5,000 B.C., and its later cultivation in other regions indicates that it spread from Mediterranean centres of domestication (Bonjean and Angus, 2001). Among today's food crops, wheat is the most abundant source of energy and protein for the world's population. It is estimated that in 2002-2003 more than 500 million metric tons of wheat were produced.

The major wheat-producing countries of the world are the United States, China, and Russia. Wheat is also extensively grown in India, West Europe, Canada, Argentina, and Australia (Food and Agriculture Organization of the United Nations, 2004). Ninety-five percent of wheat grown today is of the hexaploid type, used for the preparation of baked products. Nearly all of the remaining five percent is durum (tetraploid) wheat, which is mainly used for making pasta and biscuits (Turnbull and Rahman, 2002). The diploid wheat is rarely cultivated today although very few varieties, such as spelt, are still traded on the global market.

More than 100 million metric tons of wheat is traded annually. Kernel texture forms the fundamental basis of differentiating the world trade of wheat grain. It is common to differentiate soft and hard hexaploid wheats, and very hard durum (tetraploid) wheat as three distinct qualitative classes. Generally, the term texture is used in preference to hardness (Turnbull and Rahman, 2002; Morris, 2002). On visual inspection it is impossible for one to know the kernel texture.

World trade of wheat grain suffered for thousands of years from no reliable and direct way of measuring wheat kernel texture, thus its end-use practicability. The first instrument for estimating wheat kernel texture was designed in 1896 by measuring the force crushing the seeds (Cobb, 1896). Historically, the most practical reference method was the particle size index (PSI) method. This involves grinding a sample, and sieving a weighed amount (usually ten grams) through a

standard screen for a standard period of time. The percentage of pass-throughs is recorded as the PSI. Since soft wheat is more easily pulverized than hard wheat, it releases more fine particles on sieving, and PSI values decrease in wheat types with harder kernels (Cutler and Brinson, 1935). This grinding-time method is influenced greatly by wheat moisture content and other environmental conditions (Worzella and Cutler, 1939; Morris, 2002). Harald Perten of Tallinn, Estonia developed the Single Kernel Characterization System (SKCS) in 1993. This instrument was an important addition to the tools employed in evaluating wheat kernel texture. This device measures kernel texture by crushing one kernel between two steel surfaces. SKCS records the force required to crush the kernel and reports the average force for crushing 300 individual kernels in terms of a hardness index (HI). HI values increase with increasing kernel hardness. The SKCS now marketed by Perten Inc., Stockholm, Sweden, can complete a test in about three minutes, and simultaneously reports mean and standard deviation data for kernel weight, diameter, and moisture content, as well as the HI. The American Association of Cereal Chemists (AACC) is one of the major arbiters of international grain standards. Actually, the definition of kernel texture by the AACC was modified as a measure of the resistance to deformation on the basis of the measurement of hardness by the SKCS. Using the SKCS, hard wheat has a score of around 75 while soft wheat has a score of 30 or less (Martin et al., 1993).

C. Wheat kernel texture and its end-use

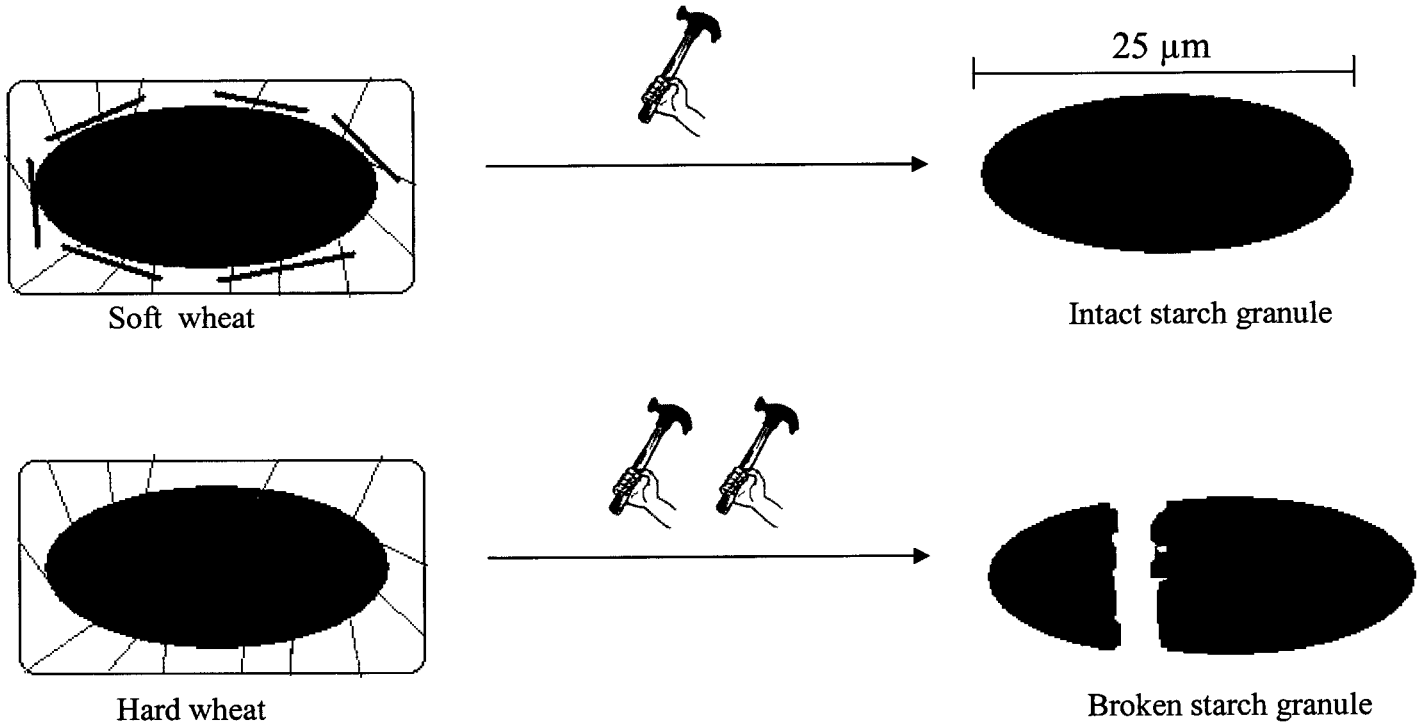
Soft wheat flour is only used for making cookies, cakes and pastries while hard wheat flour is preferred for any kind of yeast-leavened bread. The main reason for this preference is that the damaged starch granule surfaces in hard wheat afford flour an increased water uptake, yielding a moist, more expanded foam, thus tasty bread (Dubreil et al., 2002). The broken surfaces of the starch granules allow for increased hydration and gelation. The broken starch granules are also more readily hydrolyzed by alpha-amylase, which gives a greater amount of fermentable sugars for the yeast. Conversely, increased water absorption is not desired in soft wheat flour because many soft wheat products, such as short bread cookies are baked with low moisture contents (Morris, 2002). The commonly encountered all-purpose wheat flour in the market is the balanced mixture of soft and hard wheat flours to cater to the needs of customers (Leonard and Robertson, 1990; Alford and Duguid, 1995).

D. Physical basis of wheat kernel texture

Soft wheat endosperm fractures very easily with a minimum of compressive force, while hard wheat possesses an endosperm that requires a lot of compressive force before it will fracture (Morris, 2002; Turnbull and Rahman, 2002). The physical basis for endosperm hardness is the binding strength between the starch granules and protein matrix (Schofield and Greenwell, 1987) (Figure 1). In soft wheat the starch granules are thought to be more loosely attached to the protein matrix and this type

Figure 1 Model of grain textures of soft and hard wheats

This model of texture illustrates the interface between the starch granule surfaces and the protein matrix in dry mature wheat seeds. The rectangular box is a close-up view of one single starch granule embedded in the storage protein matrix of the endosperm cell's cytoplasm. Soft textured wheat flour has a lower content of broken starch granules because puroindoline is thought to transfer the crushing force through the protein matrix and away from the starch granules. Less energy is required for fracture. The starch granules are more intact. The roles of PINs during the milling process may be crucial in determining differences in adhesion/interaction between intact starch granule surfaces and the protein matrix. It is thought that in soft wheat this adhesion/interaction is weak. This model complies with the fact that PINs appear to be associated with the starch granule membranes (Dubreil et al., 2002; Morris, 2002). PINs act as a non-stick agent (lubricant) in this model instead of a glue agent (adapted from Schofield and Greenwell, 1987). In the intracellular matrix of hard wheat endosperm, not only is more force required to disrupt mature, dry structure, but the final flour is full of fractured granules, with a resulting increase in surface area for eventual water hydration by both the amorphous and crystalline zones of the integral granule.



Legend:

PINs — Interaction between starch granule surface and protein matrix

Protein matrix — Crushing force — Starch granules

of wheat is therefore easy to grind and gives fine-textured flour. In hard wheat the binding of the starch granules to the protein matrix is much stronger, and therefore more force is required to disrupt sub-cellular and cellular structure and finally to crush the kernel. This results in coarse-textured flour with bigger particle size and a higher content of broken starch granules (Schofield and Greenwell, 1987; Morris, 2002; Turnbull and Rahman, 2002).

E. Genetics of wheat kernel texture and environmental effects

Bread wheat, *Triticum aestivum* has seven pairs of chromosomes derived from three ancestral diploid progenitors (A, B, D genomes). Most wild wheats are soft and exhibit greater fungal resistance (Bonjean and Angus, 2001). It is generally accepted that hardness is a highly heritable trait. The gene that is the major determinant of kernel texture is located on chromosome 5D. This locus is called the *Ha* (hardness) locus (Jolly et al., 1996). Durum, an AABB tetraploid wheat, which lacks the D genome, including chromosome 5D, is extremely hard (Greenwell and Schofield, 1986). Recently, there was evidence that other loci on wheat chromosome 2 and chromosome 4 may also contribute, although comparably little, to grain hardness (Osborne et al., 2001). The gene loss (Gautier et al., 2000) has been confirmed by the later study (Kashkush et al., 2002).

While PINs play a key role in determining wheat flour functionality, kernel hardness is affected by a number of factors beyond genetics including nitrogen management,

tillage system, pest infestations, weather, and their interactions. Farmers always find that meeting new kernel texture standards is much more complex than merely selecting the right cultivar. While the environment can strongly affect hardness, it does not seem to affect the relative ranking of varieties, since the environment affects all varieties in a similar manner. This effect can be positive or negative depending on the environmental conditions (Lyon and Shelton, 1999; Morris, 2002). Since the precise sub-cellular macromolecular architecture of the endosperm, housing the starch granules and storage protein bodies, is still unstudied, thus a multitude of complex interactions may be involved in the mechanical phenotype of grain texture.

1.2 Puroindoline protein and kernel texture determination

A. Mechanism controlling kernel texture

The physical difference between a soft or hard wheat kernel depends on the strength of adhesion between the starch granule and the surrounding protein matrix. In soft wheat, the adhesion is weak; and fractures during milling are more likely to occur at the starch granule/protein matrix interface. This does not occur during milling of hard wheat kernels. There, kernel fracture splits open many more starch granules (Schofield and Greenwell, 1987).

In 1986, P. Greenwell and J. D. Schofield published a paper describing the first protein marker of wheat kernel texture. This marker was extracted from the water-washed wheat starch granules and separated by gradient SDS-PAGE as a

15 kDa protein. A survey of 150 different wheat varieties, including seven durum varieties established an 'unbroken' relationship: abundant 15 kDa protein associated with water-washed soft wheat starch, small amounts associated with water-washed hard wheat starch, and none associated with durum starch. Based on this finding, they proposed the 15 kDa protein does not act as glue between the starch granule surface and the protein matrix. Instead, it likely acts as a non-stick agent, which is opposite to glue. They also proposed that in the presence of low levels of the 15 kDa protein, adhesion between starch granule surface and protein matrix is strong, as shown in hard wheat. In durum wheat, in which 15 kDa protein does not exist at all due to the loss of chromosome 5D, the adhesion is the strongest. In soft wheat, the abundant presence of the 15 kDa protein abolishes the interactions between the starch granule surface and the protein matrix, making the adhesion weak or friable in comparison. Based on this relationship, they coined the name friabilin for the 15 kDa protein. The term grain softness protein (GSP) was also used by other groups to describe the same 15k Da protein to highlight its impact on kernel texture. Often many labs isolate the same proteins, so historically it bears different names, such as 3B3, GSP or tryptophanin. On the basis of this hypothesis, they predicted that hard wheat should have low or no presence of friabilin (Greenwell and Schofield, 1986; Schofield and Greenwell, 1987; Morris, 2002).

Amino acid sequencing of friabilin identified several N-terminal residues and odd internal peptide residues but none of this primary structure information alluded to a

particular function. Armstrong separated and sequenced nine soft wheat starch granule associated proteins, and found one puroindoline-like sequence (Armstrong, 1992). Later work showed that friabilin was a mixture of three or more discrete polypeptides, including PIN-a, PIN-b and GSP-1 (Jolly et al., 1993; Morris et al., 1994, Rahman et al., 1994). These proteins will be discussed in detail in the following chapters.

It was also found that this over simplified relationship could not explain the few hard grain texture wheat varieties with abundant friabilin found by large scale surveys of grains from around the world. However, Greenwell's study was the first endeavor in history to try to link kernel texture to a defined protein marker.

B. Discovery of puroindoline

In 1895, Osborne published his historical classification system of seed proteins. In this system, seed proteins are classified into four fractions on the basis of their solubility in water and various solutions.

1 Albumins: Proteins soluble in water.

2 Globulins: Proteins insoluble in water but soluble in dilute salt.

3 Glutelins: Proteins insoluble in water and dilute salt but soluble in weak acid or base.

4 Prolamines: Proteins insoluble in the above solutions but soluble in 70% ethanol.

This classical classification has not changed for more than one century and is still

widely used today (Osborne, 1895; Adeli, 1982).

In effort to discover how trace lipid can have a catastrophic effect on baking and brewing food matrices, Marion's group in Nantes along with that of Gautier's in Montpellier abandoned the century-old linear thinking of Osborne's aqueous doctrine, and tried surfactant-fishing to find new proteins (Osborne, 1895; Osborne and Mendel, 1918; Blochet et al., 1993). When studying efficacy of different non-ionic and zwitterionic detergents to extract membrane or lipid binding proteins from wheat flour, six peaks were resolved from a <20 kDa Triton X114-soluble protein fraction from wheat flour by reverse phase high-performance liquid chromatography (RP-HPLC). In fact, Triton X-114 detergent extraction of wheat flour isolates the most abundant amphiphilic protein in a nearly pure form. Two major peaks showing strong homology with each other were previously uncharacterized. The more abundant peak was sequenced entirely. The presence of a tryptophan-rich domain, consisting of five tryptophan residues among a stretch of seven amino acid residues, is rare in Nature indeed. The protein was named puroindoline to highlight the rare domain (*puro* for wheat (Greek), and indoline for the indole ring of tryptophan) (Blochet et al., 1993). Interestingly, a decade earlier a very similar amino acid sequence was contained as a predicted translation product in an oat cDNA report from Dr. Altosaar's lab (Fabijanski et al., 1988).

Later two families of wheat cDNA clones were isolated and sequenced. One clone

corresponded perfectly to the amino acid sequence of the abundant peak, which was found in the study of Blochet et al., and was named puroindoline-a (PIN-a). Another clone corresponded to the homologous minor peak and was named PIN-b. Comparison of N-terminus and peptide sequences of friabilin to those of PIN-a and PIN-b provided convincing evidence that they are the primary components of friabilin (Jolly et al., 1993; Gautier et al., 1994; Morris et al., 1994).

Puroindolines are basic cysteine-rich polypeptides, and contain a unique amphiphilic tryptophan-rich domain (Figure 2). The genes encoding PIN-a and PIN-b share 55% nucleotide similarity. The molecular mass of mature PIN-a is slightly lower than that of PIN-b (13.4 vs. 13.6 kDa). The tryptophan-rich domains are different between PIN-a and PIN-b. In PIN-a, this domain consists of five tryptophan residues and three basic residues (WRWWKWWK) whereas in PIN-b there are three tryptophan residues and two basic residues (WPTKWWKG) (Figure 2). Thus, the latter simple domain was chosen for the focus of this thesis.

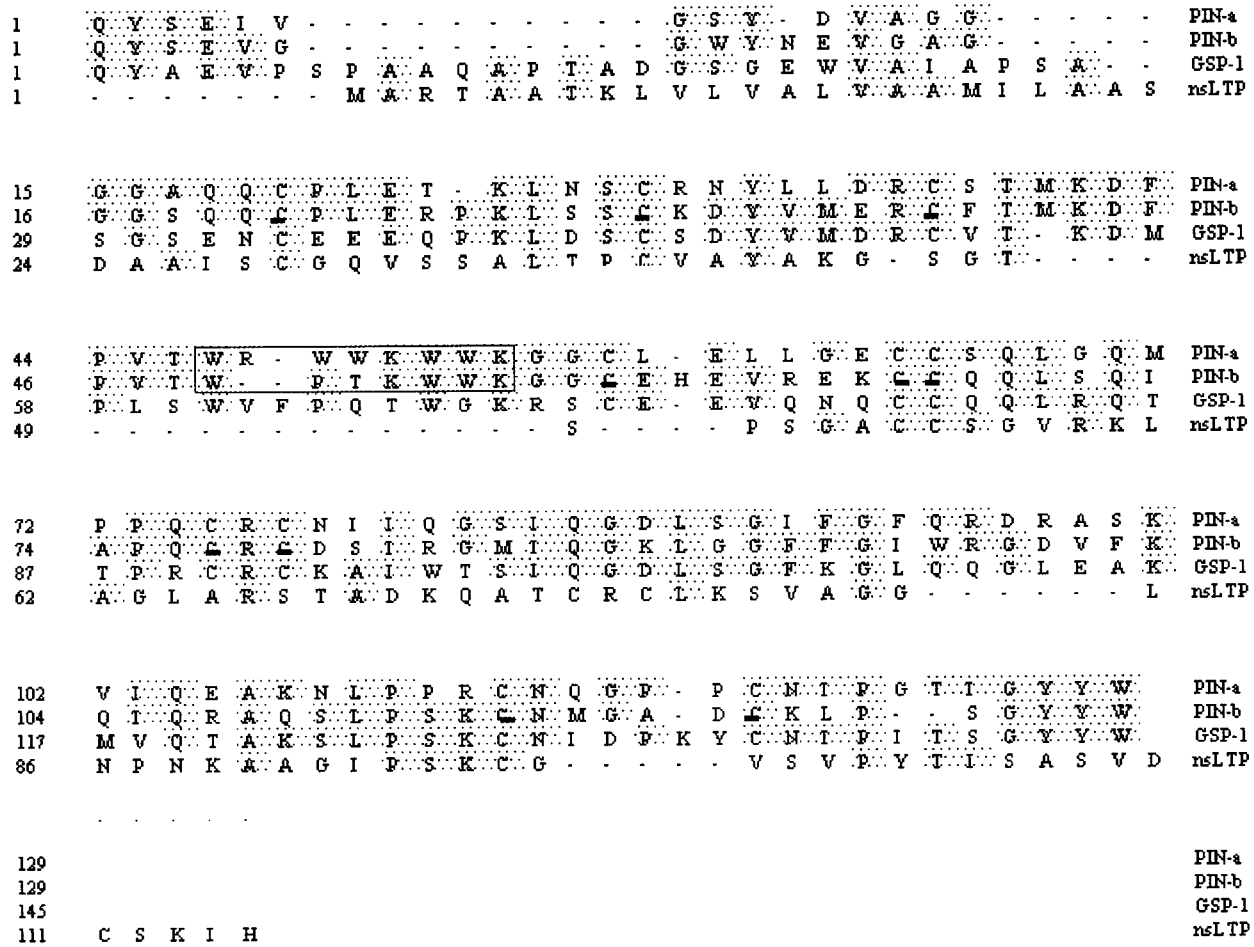
Later, GSP-1, the third component of friabilin, was reported by many different labs. GSP-1, which is also located on chromosome 5D, is closely related to the PINs. GSP is a member of the same seed cysteine –rich protein family, including alpha-amylase/trypsin inhibitors and non-specific lipid transfer proteins (nsLTP). But no direct relationship of GSP-1 with kernel texture was demonstrated (Jolly et al., 1996; Morris, 2002).

Figure 2 Sequence alignment of wheat PIN-a, PIN-b, GSP-1 and nsLTP

DNAStar (DNAStar, Inc., Madison, Wisconsin) was used to align the sequences of putative translational products of wheat PIN-a (accession number AJ302093), PIN-b (accession number AJ302102), GSP-1 (accession number AF177218) and nsLTP (accession number AF334185). Clustal W alignment method was used and all the parameters were set as default. Spaces (gaps) were introduced to generate maximum identity. The signal peptide sequences of PIN-a and PIN-b are not shown in this alignment (Gautier et al., 1994). The unique tryptophan rich domain of PIN-a and its truncated version of PIN-b are included in the blue box.

The conservative ten cysteines of PIN-a and PIN-b are underlined in blue.

Conserved amino acid residues are shaded in red. PIN-a and PIN-b proteins share 57.5% sequence identity.


1 Q Y S E I V - - - - - - - - - - G S Y - D V A G G - - - - - PIN-a
1 Q Y S E V G - - - - - - - - - - G W Y N E V G A G - - - - - PIN-b
1 Q Y A E V P S P A A Q A P T A D G S G E W V A I A P S A - - GSP-1
1 - - - - - - - M A R T A A T K L V L V A L V A A M I L A A S nsLTP
15 G G A Q Q C P L E T - K L N S C R N Y L L D R C S T M K D F PIN-a
16 G G S Q Q C P L E R P K L S S C K D Y V M E R C F T M K D F PIN-b
29 S G S E N C E E E Q P K L D S C S D Y V M D R C V T - K D M GSP-1
24 D A A I S C G Q V S S A L T P C V A Y A K G - S G T - - - - nsLTP
44 P V T W R - W W K W W K G G C L - E L L G E C C S Q L G Q M PIN-a
46 P V T W - - P T K W W K G G C E H E V R E K C C Q Q L S Q I PIN-b
58 P L S W V F P Q T W G K R S C E - E Y Q N Q C C Q Q L R Q T GSP-1
49 - - - - - - - - - - - - - - S - - - - P S G A C C S G V R K L nsLTP
72 P P Q C R C N I I Q G S I Q G D L S G I F G F Q R D R A S K PIN-a
74 A P Q C R C D S I R G M I Q G K L G G F F G I W R G D V F K PIN-b
87 T P R C R C K A I W T S I Q G D L S G F K G L Q Q G L E A K GSP-1
62 A G L A R S T A D K Q A T C R C L K S V A G G - - - - - - - L nsLTP
102 V I Q E A K N L P P R C N Q G P - P C N I P G T I G Y Y W PIN-a
104 Q I Q R A Q S L P S K C N M G A - D C K L P - - S G Y Y W PIN-b
117 M V Q T A K S L P S K C N I D P K Y C N I P I T S G Y Y W GSP-1
86 N P N K A A G I P S K C G - - - - - V S V P Y T I S A S V D nsLTP
129 PIN-a
129 PIN-b
145 GSP-1
111 C S K I H nsLTP

C. Mutations and engineering of kernel texture

Soon after the discovery of friabilin, labs around the world struggled to understand the puzzle of friabilin: Substantial differences in friabilin levels were identified in water-washed starch between soft, hard and durum wheat kernels, but similar levels of friabilin were observed in the native endosperm of soft and hard wheat (Morris, 2002). The first reports that a mutation in the tryptophan-rich domain of the PIN-b highly correlated with grain texture were published by the Morris group (Giroux and Morris, 1997; 1998). Later, other mutations were characterized (Table 1). They designated the soft puroindoline allele sequences as *Pina-D1a* and *Pinb-D1a* to comply with the revised guideline for Nomenclature of Biochemical/Molecular Loci in Wheat and Related Species (Morris, 2002). The most common allele mutation in hard texture wheat in North America is designated as *Pinb-D1b* (Gly56 to Ser56 mutation) (Giroux and Morris, 1997).

Each of these mutations was proposed to cause a 'loss-of-function' of PIN-b, resulting in a hard wheat kernel texture. It was also found that some hard wheat varieties did not contain mutations of PIN-b. Instead, these hard wheat varieties lacked the PIN-a transcripts, indicating PIN-a was also linked to kernel texture. Absence of PIN-a or the presence of 'loss-of-function' mutation in PIN-b was required for the hard kernel texture, while both functional PIN-a and PIN-b were required for the wild type soft wheat kernel texture (Giroux and Morris, 1998). There was evidence that soft textured wheat was associated with the presence of both

Table 1: Current known 'hard' mutations of puroindolines

PIN-a	PIN-b	Molecular Change	Phenotype	Reference
Native	*Native*	*No change*	*Very soft (oat)*	Fabijanski., *1988*
native	Native	No change	Soft, wild type (wheat)	Giroux and Morris1997
mutant	Native	Unknown	Hard, PIN-a null (wheat)	Giroux and Morris 1998
native	Mutant[a]	Gly56 to Ser56	Hard, PIN-b point mutation (wheat)	Giroux and Morris 1997
native	Mutant	Leu70 to Pro70	Hard, PIN-b point mutation (wheat)	Lillemo and Morris 2000
native	Mutant[a]	Trp54 to Arg54	Hard, PIN-b point mutation (wheat)	Lillemo and Morris 2000
native	Mutant	Trp49 to stop codon	Hard, PIN-b null (wheat)	Morris et al., 2001
native	Mutant[a]	Trp54 to stop codon	Hard, PIN-b null (wheat)	Morris et al., 2001
native	Mutant	Cys66 to stop codon	Hard, PIN-b null (wheat)	Morris et al., 2001
native	Mutant[a]	Lys55 to Glu55	Hard, PIN-b point mutation (wheat)	Pan et al., 2004

a: Any amino acid residue mutation in the very short (Trp54-Lys55-Gly56) region will change the wheat kernel texture from soft to hard, suggesting that this region may be part of the functional domain of PIN-b, and awaits further study.

native puroindoline proteins and not the total puroindoline content (Hogg et al., 2004). It was suggested that either the loss of PIN-a expression or the PIN-b 'loss-of-function' mutation might alter the lipid binding capacity of the PIN complement. This might affect the way starch granule membranes collapse and starch granules coalesce during seed desiccation, which finally alters the dry kernel texture (Gibbon et al., 2003). The kernel texture is caused by a mechanism that relies mainly on the interaction between the starch granules and the surrounding protein matrix. This hypothesis has not been tested directly (Schofield and Greenwell, 1987; Giroux and Morris, 1998; Morris, 2002).

Anthesis is the developmental period when floral parts open and pollen is dispersed from the anthers upon dehiscence. This is the period during which fertilization occurs and seed endosperm growth commences. Some contradictory evidence suggested that the endosperm texture phenotype could be clearly detected by scanning electron microscopy in very young developing seeds from five days post-anthesis (dpa), while the puroindolines were only detectable after 10–15 dpa (Turnbull et al., 2003). There were very few starch granules present in the wheat endosperm and no protein deposits were visible at 5 dpa. Thus there may be a difference between the timing of puroindoline accumulation and the development of grain texture, which weakens the above hypothesis. Certainly, having a high-resolution three-dimensional (3D) structure of a puroindoline molecule would go a long way to clarify the 'kernel of truth'. Hence, preparation of high quality

PIN-b sample for structural determination is one of the goals in this thesis.

The discovery of PIN and its impact on kernel texture shed light on the possibility of engineering grain kernel texture. 'Going soft' is not necessarily bad for improving a crop's quality. One of the most vitreous, brittle or glassy grain textures known is that of rice (*Oryza sativa*). Shattered kernel represents a huge loss in the rice grain polishing industry. Rice which is *pin* null, was transformed with wheat native type *pin-a* and *pin-b* (Krishnamurthy and Giroux, 2001b). Expression of the transgene(s) reduced rice kernel hardness as evidenced by indirect proof. The force needed to crush kernels was reduced when the transgenic rice seeds were ground.

The amount of starch granule damage was decreased, which probably was due to less force needed to loosen the starch granule from the endosperm. The applied force to crush the transgenic rice seeds was thought to be transmitted multi-directionally throughout the endosperm cell contents, for example, tempering the glass-natured or vitreous normal rice seeds into a more powdered-cake form. The softest transgenic rice line expressed both PIN-a and PIN-b (Krishnamurthy and Giroux, 2001b).

As an aside, Tamar Ferreira (a 2002 summer student in Dr. Altosaar's lab) and I adapted the Perten SKCS instrument, which was designed for direct wheat kernel texture measurement, for rice measurement. The above results were confirmed by direct transgenic rice kernel texture measurement, which support the hypothesis that the new presence of PIN softened the grain texture. Besides rice, knock-in transgenics have also been tried in wheat itself. *Pinb-D1b* mutation, most common

in hard textured wheat varieties in North America, was 'fixed' by the expression of wild-type *Pinb-D1a* protein in genetically transformed plants. Transgenic wheat seeds from the hard parental line, expressing wild-type PIN-b, were soft kernel in phenotype (Beecher et al., 2002a; Hogg et al., 2004).

D. Current knowledge of 3D structure of puroindoline

Understanding the biological function of any protein will benefit from knowing its three dimensional (3D) structure. Also it is crucial to know the 3D structure if this 'hard' question is to be answered: How do single amino acid mutations make bread possible? Unfortunately, the current knowledge of puroindoline structure is very limited. Infrared spectroscopy indicated that PIN-a and PIN-b have similar secondary structures of approximately 30% alpha-helix, 30% beta-sheet, and 40% unordered structure at pH 7. Combined infrared and sequence conformational analysis assigned a secondary structure of four helical bundles connected with flexible loops, which is similar to the structures of plant nsLTPs (Le Bihan et al., 1996). In this model the tryptophan-rich domain is included in the loop between helix one and two, and is probably in a beta-sheet conformation. PINs exhibit a similar folding pattern to that of plant nsLTPs. Based on the sequence and secondary structure similarity, Lillemo suggested a very preliminary 3D model using a homology-based 3D structure prediction algorithm (http://arken.nlh.no/~ipfmol/genetics.html; content as of March 31, 2005). However this technique demands high homology (current limit is at least 50%)

between target protein and protein with known solution or crystal structure, so it is impossible to have an accurate PIN structure without first resolving it experimentally.

E. PIN homologues in other species

Leaving wheat aside for a moment, PIN-like genes are present in closely related cereals: barley, oat, rye, but absent in more distantly related grasses: maize, rice and sorghum (Gautier et al., 2000). PIN-like sequences in barley, oat and rye genomes are highly conserved. As early as 1988, our laboratory reported the sequence of a cDNA for a major storage protein in oat, which was later shown to be related to wheat PIN (Fabijanski et al., 1988; Blochet et al., 1993; Tanchak et al., 1998).

Interestingly, the tryptophan-rich region is similar to a bovine defensin, indolicidin (Del Sal et al., 1992; Selsted et al., 1992; Staubitz et al., 2001; Schibli et al., 2002). The amino acid sequence alignment is shown in Figure 3. Many antimicrobial peptides are important in food processing, which may suggest that PIN has potential functionality in the food industry (Cleveland et al., 2001).

In barley, a major quantitative trait locus for kernel texture is located on the short arm of chromosome seven. PIN protein homologues have been separated from the surface of barley starch granule (Darlington et al., 2000, 2001; Beecher et al., 2002b).

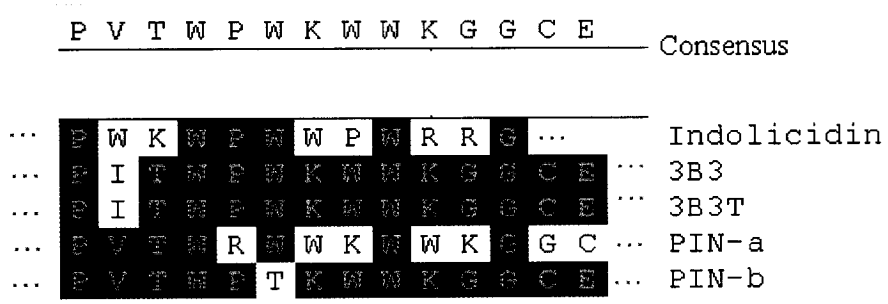
Figure 3 Local sequence alignment among PINs from wheat, oat and bovine indolicidin

Amino acid residues in red shade match the consensus sequence exactly.

The bovine indolicidin is thought to play a role in mammalian innate defense system (Del Sal et al., 1992; Selsted et al., 1992; Staubitz et al., 2001; Schibli et al., 2002).

The diagnostic tryptophan-rich domain is the hallmark of the PIN gene family.

The PIN gene family from wheat (PIN-a and PIN-b) and oat (3B3 and 3B3T) share strong local similarity with indolicidin in the tryptophan-rich domain (Blochet et al., 1993; Tanchak et al., 1998).


P V T W P W K W W K G G C E
Consensus
Indolicidin
3B3
3B3T
PIN-a
PIN-b

Among the three soft and three hard textured barley varieties used in the study, all contained proteins homologous to wheat PINs, but PCR analysis did not reveal any difference in the deduced amino acid sequences similar to the mutation/texture relationships found in wheat (Table 1). In barley no clear relationship can be established between the presence of PIN homologues and grain texture (Darlington et al., 2001). One explanation is that barley exhibits relatively little texture variation. All the barley varieties used in this study appear to behave in a fashion similar to hard wheat. Other genes or environmental effects contributed to the limited texture variation observed in the barley varieties used in their limited scale screening. The possibility that a 'super' soft barley with a different amino acid sequence of a PIN homologue may be found by large scale screening of world genotypes cannot be ruled out (Beecher et al., 2002b). The world cannot live (drink) by hard barley alone!

PIN homologues have not yet been separated and sequenced from oat or rye. PIN homologue proteins have been observed in oat kernels with immnuofluoresence microscopy and immunoblot using different anti-PIN antibodies (Mohammadi et al., personal communication). Rice has the hardest kernel texture among all the major cereal crops. They are devoid of PINs, providing a stark contrast with oat (Tanchak et al., 1998; Gautier et al., 2000). Oat has the softest kernel texture and contains the most abundant level of PIN-like proteins in seeds (Tanchak et al., 1998). This suggests strongly that PIN homologues may play an important role in oat kernel

texture determination. A harder oat variety would permit a more economical milling to produce oat bran and oat flour streams.

1.3 Functions of puroindolines

A. Antmicrobial protein/peptide: natural born killers

Plants are exposed to a large number of pathogenic fungi. Although they do not have a genuine immune system like mammals, plants have evolved a variety of potent defense mechanisms. One strategy is synthesis of low-molecular-weight compounds, peptides and proteins that have antifungal activity (Ganz, 2003).

These proteins/peptides appear to be involved in either constitutive or induced plant defense systems against fungal attack. Interestingly, most of the identified plant antimicrobial proteins/peptides (AMPs) contain even numbers of cysteines (4, 6, 8 or 10). These cysteines are all pairwise connected by disulfide bonds, providing high stability to the peptides (Selitrennikoff, 2001).

AMPs are evolutionary ancient self-defense weapons (Boman, 1991). They have been identified in almost every species throughout the plant and animal kingdoms, suggesting they play an indispensable role in successful evolution. AMPs are ancient, but not outdated. They remain effective against large varieties of bacterial pathogens. The success of AMPs challenge the general dogma that microbes can and will develop resistance to any substance. The diversity of AMPs is so

tremendous that the early classification on a taxonomical basis is inadequate (Zasloff, 2002). Alternatively, the classification based on chemical-structural considerations has proved more useful (Andreu and Rivas, 1998). This classification defines two broad groups, corresponding to linear and cyclic structure, respectively (Table 2). It is interesting that PIN-b is among the very few proteins fitting the definitions of both sub groups of linear AMP (Table 2). The study of PIN-b may serve as a model to understand other AMPs.

B. Fungicidal activity of PIN

Native PINs extracted from wheat have demonstrated fungicidal activity against various pathogens (Dubreil et al., 1998; Krishnamurthy et al., 2001a). To evaluate the efficacy, a test system has to be established. Bioactive PIN should confirm a correct folding pattern and maintain a stable native conformation. Any purified PIN, which is shown positive in a bioassay, will be useful for protein structure determination by biophysical methods (NMR, X-ray crystallography, circular dichroism, atomic force microscopy, etc). In this thesis, two fungal pathogens were therefore chosen for their significant roles in fungal diseases.

Verticillium wilt is caused by the pathogen *Verticillium dahliae*. This fungal disease causes stunting, wilting, early senescence and total plant death. The disease is most severe during autumn-winter. A considerable decrease in yield, if infected by this pathogen, is expected, but, sometimes, without damage to visual quality.

Table 2 The chemical-structural based classification of AMPs

Category	Structural hallmarks	Examples and references
Linear AMP	alpha-helical amphipathic conformation	Andropin (Samakovlis et., 1991) Bombinin (Mignona et al., 1993)
	unusual composition, rich in Pro, Arg, Trp	PR39 (Zhang, et al., 1997) Indolicidin (Selsted et., 1992)
Cyclic AMP	single or multiple disulfide	Thanatin (Fehlbaum,et al., 1996) Pipinin (Morikawa, et al., 1992)

This classification system was first proposed by Andreu and Rivas (Andreu and Rivas, 1998). In 843 identified AMPs, 40% have one or more disulfide bonds, suggesting these bonds may be crucial in bactericidal activity (Tossi et al., 2000). PIN fits the definitions of both sub groups of linear AMP.

Fusarium culmorum, a soil-borne fungus, is widespread and persists several years in soil. It causes serious wilting of watermelon, muskmelon, cucumber, squash and other vine crops, while also being a problem for wheat (Ingram and Robertson, 1999). In vitro assays indicated native PIN-b had minimal inhibitory concentrations (MIC) against *Verticillium dahliae* and *Fusarium culmorum* of 30 μg/mL and 40 μg/mL, respectively (Dubreil et al., 1998). So the MIC bioassay was also used in this thesis to monitor the native conformation of any purified PIN-b preparations.

C. Can PIN be a food preservative?

Despite many preservation techniques available, the spoilage and poisoning of foods by microorganisms is a problem that has not yet been brought under adequate control. For example, it is estimated that the cost of food-borne illnesses in the USA alone is from 2.9 to 6.7 billion dollars per year (Powell and Attwell, 2000).

Considering the recent trend that consumers are increasingly avoiding foods prepared with preservatives of chemical origin, natural alternatives are therefore needed to achieve a sufficiently long shelf-life of foods and a higher degree of safety against food born pathogenic microorganisms. PIN may be an ideal candidate for a natural preservative. PIN genes are only found in major cereal seeds, which have already proven their safety in our daily food supply over thousands of years. To test the concept that natural food proteins can serve as antimicrobial agents in ready-to-eat food preservation, a large quantity of functional PIN is required. Development of a cost-effective system to produce PIN-b, hence, is a major goal of this thesis.

D. Technical implications of PIN in brewing

In beer quality, the ability to form a stable head of foam when beer is poured and to achieve characteristic flavour are important factors. Lipid content of beer can affect both factors. Oxidation of lipids contributes to off-flavor development, while lipid interactions with the protein film/matrix stabilizing the gas bubbles of foam are thought to cause the 'head' collapse. Beer lipids, which originate from barley malt or yeast, with levels as low as 0.2-0.4 μg/mL can ruin the beer quality.

Market beers are often returned because of the problems with off-flavors.

Any improvement to reduce off-flavors and enhance foam formation will contribute financial benefits to the beer industry (Sorensen et al., 1993). Despite its importance in determining beer quality, the state of lipid in beer (i.e. whether lipid is free or bound to proteins) and how that state affects quality are still unknown.

Although the mechanism is poorly understood, there is evidence that PINs can aid the recovery of beer foam that has been adversely affected by lipid. Other proteins, such as nsLTP have also been shown to play a role in beer foam formation (Douliez et al., 2000). These data hinted that protein-bound lipid is not as damaging to foam formation as free lipid. The effect of protein binding on lipid oxidation is still unknown. This also suggests that PIN/nsLTP may be relevant to other food systems where protein-lipid interactions play an important role in determining foaming properties, such as in bread-making or off-flavour development in dairy products (Dr. Peter Wilde, personal communication). So understanding PIN structure and

function will not only help critical areas of food and nutrition, but may also contribute to the unpredictable areas of colloid and material science as well as in lipid transport and metabolism in mammalian regulatory systems. Not only may native PIN structures prove useful in novel applications, but mutant PINs may also shed light on future innovations.

1.4 Sample requirements for puroindoline in this project

A. NMR is a suitable method to study the 3D structure of PIN

In the past ten years, NMR has proven to be an alternative to X-ray crystallography for the determination of macromolecular structure. The fundamental characteristic of this technique is that in the presence of a strong magnetic field, there is a small population difference between NMR-active nuclei in high energy and low energy states, permitting absorption of radiofrequency radiation. It has the unique ability to accurately measure the dynamic properties of proteins and to probe the process of protein folding. However, a major drawback of protein NMR is its size limitation. The current size limitation for structure determination by NMR is about 35 kDa (Yu, 1999). Larger proteins have slower tumbling rates and shorter NMR signal relaxation times, which usually reduce the sensitivity of the complicated pulse sequences. A second reason is that the more amino acid residues a protein has, the more complex its spectrum, simply because there are more signals and more interactions among them. PIN-b, fortunately, is a small protein (129 amino acid residues) and this current size limit of NMR is, hence, not a major concern of

resolving this protein.

NMR may be a better technique than X-ray crystallography for the structural determination of PIN-b. X-ray crystallography requires large, stable protein crystals. However crystallizing proteins can be challenging and often presents the biggest bottleneck of this technique (Guss and King, 2000; McPherson, 2002). PIN-b has a hydrophobic tryptophan rich domain, which may be flexible in solution (Lillemo, 2001). This hydrophobic and flexible domain might make PIN-b crystallization particularly difficult since structural heterogeneity can prevent crystal formation. For this reason, I decided to avoid crystallization of PIN-b and use solution NMR methods instead. .

There is also a possibility that the flexible tryptophan-rich loop, which is the most unique domain in puroindoline, may fail to be resolved by using X-ray crystallography. NMR has the unique advantage of possibly being able to examine the dynamics of this flexible loop. It might prove possible to resolve an average structure for the tryptophan-rich loop using NMR although its flexible nature may impede interpretation of a unique conformation (Cavanagh et al., 1996). Also this technique has the possibility to study the interaction of PIN-b with solvents mimicking cell membranes (Guss and King, 2000).

Usually proteins are investigated by NMR at low pH since the amide protons undergo rapid base-catalyzed exchange with the bulk water at neutral pH.

For this reason, a protein sample subject to NMR experiments should maintain a folded state under lower pH conditions, which can slow amide exchange.

In general lower salt conditions tend to be favorable for NMR experiments. .

For protein NMR, samples should be of >95% purity and in concentrations of >1 mM. Considering the suitability of NMR to resolve the 3D structure of PIN-b, one of the goals of my thesis is to produce enough quantity of PIN-b protein suitable for NMR.

B. Expression systems of PIN

i. Expression of isotope labeled protein in mammalian and baculovirus systems

The natural abundances of NMR active isotope ^{13}C and ^{15}N are only 1.1% and 0.36% respectively. This makes it necessary to isotope label proteins larger than 10 kDa (Marion et al., 1989; Cavanagh et al., 1996; Goto and Kay, 2000).

The incorporation of stable isotopes of ^{13}C and ^{15}N into protein is regularly achieved by growth of a suitable expression host in a medium containing biosynthetic precursors for both amino acids and nucleotides. The currently tested protein expression systems for this purpose are listed in Table 3. Considering the prohibitive cost of isotope labeled media for mammalian or insect cell

Table 3 Rough comparison of current isotope-labeling protein expression systems

	Expression level	Isotope labeling price	Post-translational modifications
E. coli[a]	High	Low	No
Yeast[b]	Medium to High	High	Some possible
Cell-free system	Medium to High	Very High	Some possible
Mammalian cell line	Medium	Very High	Possible

a: *E. coli* is the most popular expression host.

b: Yeast has also been used as an expression host, but in far fewer instances.

lines, few isotope labeled proteins were expressed using these two host systems. *E. coli* is the most popular expression host. Yeast has also been used as an expression host, but in far fewer instances.

ii. Expression of PIN-a in methylotrophic yeast (*Pichia pastoris)*

PIN-a is the closest homologue of PIN-b in nature. They share 57.5% sequence identity (Figure 2) and both of them are wheat kernel texture determinants and fungal killers. It is reasonable to presume that a successful PIN-a expression system may be helpful to express PIN-b. Indeed, soluble recombinant PIN-a expression had been achieved, using a methylotrophic yeast system (*Pichia pastoris*). Their yield was 14 mg/L and 80% of the PIN-a was soluble under optimized conditions using 226 g/L of methanol (Issaly et al., 2001). Considering the cost of ^{13}C isotope for such a methanol supply, the methylotrophic yeast system was not deemed desirable for our PIN-b production. As was suggested in Table 3, each attempt at one liter batch would cost $29,000 as a minimum for ^{13}C /^{15}N isotopic chemical supply. One of the goals of this thesis sets out to manufacture isotope labeled PIN-b in a cost-effective way, which maximizes the amount of isotopic protein sample obtained from one litre of isotope-enriched media.

iii. Functional expression of disulfide-rich protein in *E. coli*

As discussed in Table 3, *E coli* is the most convenient and economical host to express isotopic protein. The basic approach is to use the standard techniques of molecular biology to construct a plasmid, known as an expression vector. The expression

vector contains the gene of interest placed behind a promoter that stimulates constitutive gene expression by the organism's RNA polymerase. A large quantity of the desired recombinant protein is required because the NMR experiments require protein samples to have a concentration of approximately 1 mmol/L to produce sufficient sensitivity. The key to incorporating ^{13}C and ^{15}N into the over expressed protein is to feed the bacterium with restrictive nutrients, in which the sole carbon and nitrogen source contains > 98% ^{13}C and ^{15}N, respectively. The most commonly used and cheapest reagents for this purpose are ^{15}N-NH_4Cl and ^{13}C-glucose. In addition to these two isotopic chemicals, *E. coli* requires phosphate, sulfate (for DNA and protein synthesis), magnesium, calcium (for co-factor of enzymes) and other trace elements to sustain its life, and NaCl is routinely added to the medium. In some cases, additional supplements are advantageous, such as vitamins (thiamine hydrochloride in particular) and heavy metals (Muchmore et al., 1989; Venters et al., 1991).

Folding is the process whereby a newly synthesized polypeptide chain acquires its 3D conformation. Usually the three-dimensional structure of a protein is stabilized by the formation of disulfide bonds between spatially adjacent cysteine residues. Historically, the most important proof was the in vitro renaturation of denatured and reduced bovine pancreatic ribonuclease A (RNase A) into a native, biologically active enzyme through manipulating the four disulfide bonds (Anfinsen et al., 1961; 1972 Nobel Prize in chemistry laureate). The cytoplasm of *E. coli* is a reducing

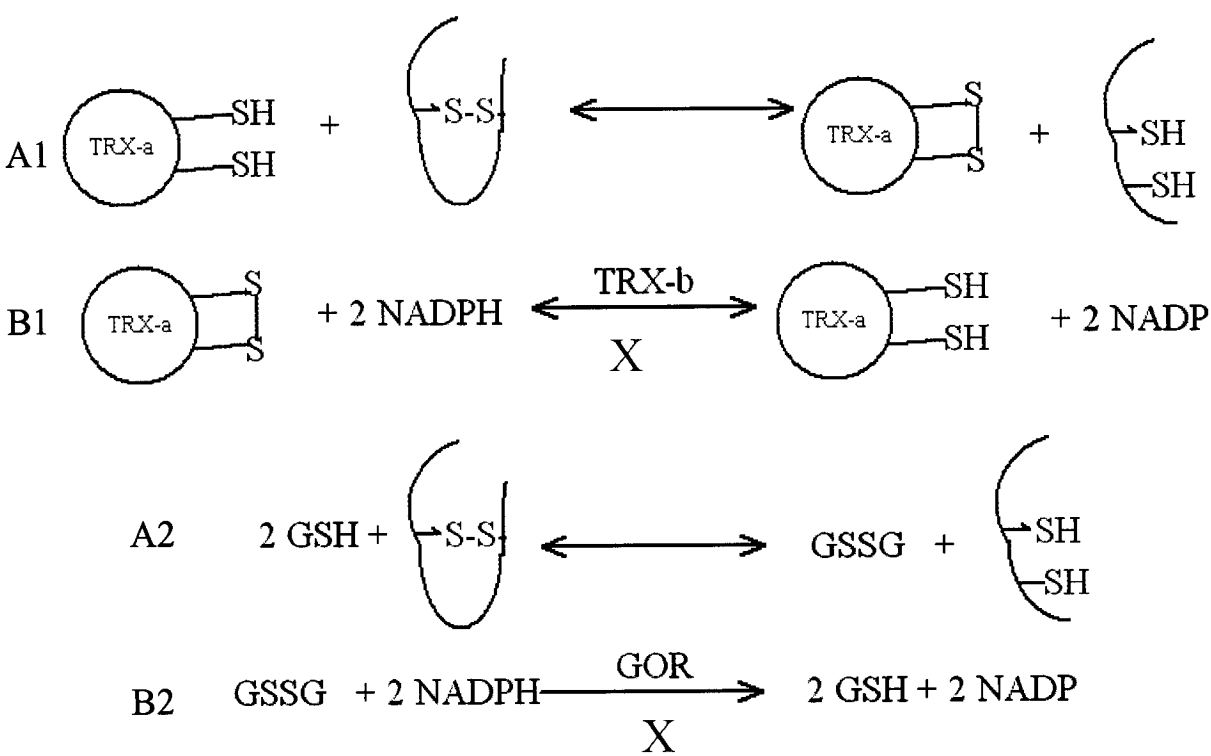
environment where cysteines do not engage in disulfide bonds. Any disulfide bond is rapidly reduced through the action of a major disulfide reducing protein, such as thioredoxin-a (TRX-a). TRX-a is kept reduced by thioredoxin reductase (TRX-b), which is a specific TRX-a reductase. Other disulfide reducing enzymes, for example glutaredoxin, also play important roles in this process (Rietsch and Beckwith, 1998).

TRX-a is a 12 kDa protein, which is found in virtually all organisms at various abundances. TRX-a is implicated in a variety of physiological roles. Its major role is, in general, as an efficient hydrogen donor for the reduction of sulfoxide (Pigiet and Schuster, 1986). Like TRX-a, glutathione is also found in virtually all organisms. Glutathione (GSH) also reduces protein disulfide bonds. It is catalytically reduced to GSH by the glutathione oxidoreductase (GOR).
The schematic diagram shows the reactions that TRX-a and GSH/GSSG undergo in the cytoplasm (Figure 4) (Stewart et al., 1998). If PIN-b is expressed in the reductive cytoplasm of *E. coli*, its disulfide bonds are expected not to form correctly.

Considering the benefits of *E. coli* expression system (Table 3), it is advantageous to explore the possibility to express PIN-b with proper disulfide formation in genetically engineered *E. coli* strain, which may have an oxidative cytoplasm. Recent data revealed that in Origami™ strain (*trxB* and *gor* double knock out), the

Figure 4 Classical assumption of the roles of thioredoxin (TRX-a) and glutathione (GSH/GSSG) during disulfide bond formation in *E. coli* cytoplasm

Once formed, the target disulfide bond in a newly synthesized protein (shown in red) can be reduced by TRX-a (A1) or GSH (B1). Reduced state TRX-a and GSH are recycled by TRX-b (A2) or GOR (B2). Under physiological conditions, the *E. coli* cytoplasm is maintained in a reduced state that strongly disfavors the formation of stable disulfide bonds. Both *trxB* and *gor* genes are knocked out in *E. coli* Origami ™ strain. Red crosses indicate that the pathways to recycle the TRX-a and GOR are blocked in this strain (Pigiet et al., 1986; Rietsch and Beckwith, 1998; LaVallie et al., 2000; Lauber et al., 2001; Ritz et al., 2001; Bellinzoni et al., 2002; Kadokura et al., 2003). GOR stands for glutathione oxidoreductase.


A1
TRX-a
SH
SH
+
S-S
TRX-a
S
S
+
SH
SH
B1
TRX-a
S
S
+ 2 NADPH
TRX-b
X
TRX-a
SH
SH
+ 2 NADP
A2
2 GSH +
S-S
GSSG
+
SH
SH
B2
GSSG + 2 NADPH
GOR
X
2 GSH + 2 NADP

TRX-a serves as oxidant instead of its normal role as protein reductant (Stewart et al., 1998). In this mutant, expression of wild-type TRX-a tag has a modest to large effect to enhance the formation of disulfides in the cytoplasm (Prinz et al., 1997; Bessette et al., 1999; Ohnuma et al., 2002; LaVallie and Collins-Racie, 2003). This mutant host is best teamed up with the pET-32 vector to express cysteine-rich heterologous protein since the thioredoxin fusion tag further enhances the formation of disulfide bonds in the cytoplasm (Origami™ manual, EMD Biosciences, Inc., San Diego, California).

One possible reason for choosing such a plasmid-cell match-up is that TRX-a and glutathione are kept in the oxidized state in this double knock-out host (*trxB*$^-$/*gor*$^-$). They do not reduce the disulfide bonds in the newly synthesized target proteins. And the expressed TRX-a tag moves the reaction in Figure 4A towards the direction of favoring disulfide bond formation in the target protein.

This mechanism can easily explain the above reports that pET-32b/Origami™ is a disulfide bond favorable vector/host combination. But the naturally occurring pathways for protein disulfide bond formation/reduction are not that simple. Although not fully understood, other multiple pathways also contribute to native disulfide bond formation/reduction (Rietsch and Beckwith, 1998; Ritz and Beckwith, 2001; Kadokura et al., 2003). The detailed discussions of these pathways and their cross-talks are beyond the scope of this thesis. Only the dynamic

thioredoxin-glutathione redox system was utilized to promote and enhance a properly folded puroindoline-b in *E. coli* cytoplasm.

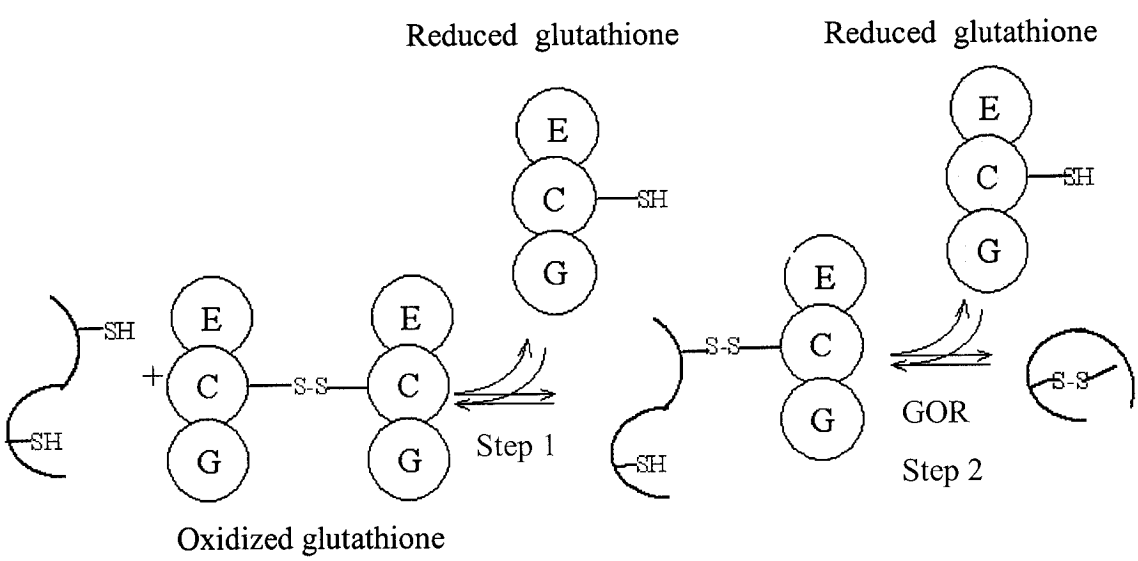
C. Cocktails facilitating disulfide bond formation

Although the above plasmid-cell match-up (pET-32/Origami™) has successfully been exploited to express several disulfides rich proteins, still many proteins could not be expressed with proper disulfide bonds. In these cases, a tailored redox buffer system is necessary to manipulate disulfide formation in vitro. It is well-known that an appropriate GSSG/GSH redox balance can provide sufficient oxidizing equivalents for native disulfide bond formation in vitro (Lyles and Gilbert, 1991). Similarly, in vitro TRX-a can catalyze the formation and the rearrangement of protein disulfide bonds (Watt et al., 1997). In vitro TRX-a must be in oxidized form to effectively promote disulfide bond formation, and the oxidized form of TRX-a is required. Addition of oxidant enhances the rate of refolding by generating the oxidized form of TRX-a (Pigiet and Schuster, 1986).

RNAase A is a classical model to study protein disulfide bond formation and refolding. A two-step mechanism is well established (Hantagan et al., 1974; Rothwarf and Scheraga, 1993). The first step is the rapid formation of disulfide bonds (Figure 5). GSSG effectively catalyzes this step by forming stable mixed disulfide intermediates. TRX-a is less effective in this step because the second thiol in the active site (Trp-Cys-Gly-Pro-Cys-Lys) may compete with target thiols to form

Figure 5 In vitro model of glutathione and thioredoxin catalyzed disulfide-bond formation

Disulfide bond oxidation and isomerization are thought to proceed via a mixed disulfide intermediate between the TRX-a or GSSG and the substrate protein (adapted from Lundstrom et al., 1992). TRX-a is more potent in the second step. It promotes disulfide interchange, like disulfide isomerase C (DsbC).


Reduced glutathione
Reduced glutathione
E
C
SH
G
SH
E
C
S-S
G
E
C
G
Step 1
S-S
C
G
SH
GOR
Step 2
S-S
Oxidized glutathione

an intramolecular disulfide. This hetero-dimer intermediate usually is not stable, due to the steric hindrance. TRX-a is efficient to catalyze the second step of the process, known as disulfide interchange. TRX-a plays a role like protein disulfide isomerase DsbC (Hantagan et al., 1974; Maskos et al., 2003). TRX-a under optimized condition promotes disulfide formation efficiently (Pigiet et al., 1986; Lundstrom et al., 1992).

But the conditions for in vitro folding have to be studied on a case by case basis. Some parameters such as, pH and GSSG:GSH concentrations need to be optimized especially at multi-milligram or bigger manufacture scale (Monthony et al., 2003). In this thesis, thioredoxin-glutathione redox buffer system was utilized to facilitate PIN-b disulfide bond formation in vitro.

1.5 Summary of research interests

The hypothesis of this thesis is that the ten cysteine containing PIN-b can be refolded in vitro using tailored redox buffer. The rationale is that the thioredoxin and glutathione are proven reagents that have yielded properly folded proteins, so they should aid in PIN-b folding as well. The goals of this project include cloning PIN-b from wheat, expression vector construction, recombinant protein expression, solubilization, disulfide bond facilitation and bioassay.

Chapter II Materials and Methods

2.1 Molecular cloning of PIN-b

A. Wheat genomic DNA extraction

Wheat (*Triticum monococcum*) seeds (accession number: CN 38852) were obtained from Plant Gene Resources of Canada, Agriculture and Agri-food Canada (Saskatoon, Saskatchewan). A protocol slightly modified from the original method (Kang et al., 1998) was used to extract the genomic DNA from wheat seeds. After removing the hull, one seed was pulverized by a hammer blow, and the crushed seed was placed into a 1.5 mL micro centrifuge tube. DNA extraction buffer (500 µL) (see Appendix for all buffer recipes) was added, and the tube was incubated at 37 °C for one hr. After adding 400 µL of cetyltrimethylammonium bromide (CTAB) solution, DNA was gently extracted using chloroform:isoamyl alcohol (24:1) with 5% phenol. The tube was centrifuged at 16,000 *g* at 4 °C for 10 min. The supernatant was transferred into a new tube. Isopropanol (200 µL) was added to the supernatant to precipitate DNA. Incubation at 37 °C for 15 min was followed by centrifugation at 16,000 *g* at 4 °C for 20 min. The supernatant was discarded, and the pellet was washed with ice-cold 70% ethanol twice, air-dried, and resuspended in 15 µL of tris-EDTA (TE) buffer.

B. Amplification of PIN-b coding sequence by polymerase chain reaction

All enzymes unless specified were purchased from New England Biolabs Inc.,

(Beverly, Massachusetts). All plasmid vectors were purchased from EMD Biosciences Inc., San Diego, California. The forward and reverse primers for PIN-b coding sequence amplification contained the restriction sites for *Nco* I and *Xho* I, respectively (Alpha DNA Inc., Montreal, Quebec). The sequences were as follows: (forward primer) 5' cgg agc cat ggc aca ata ctc aga agt t 3' and (reverse primer) 5' acg acg gct cga gtc acc agt aat agc cac t 3' (Mullis and Faloona, 1987). The recipe for the PCR mixture was: 0.1 mM dNTP, 0.5 mM forward and reverse primer, 1 μL wheat genomic DNA, 1 unit *Pfu* polymerase (Stratagene Inc., La Jolla, California), 20 mM tris (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin (BSA). Total reaction volume was 50 μL.

The parameters for the PCR thermocycler (Mastercycler Personal, Brinkmann Instruments Inc., Westbury, New York) were as follows. After hot start at 94 °C for 3 min, 30 amplification cycles were repeated for these three steps: 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 30 sec, and the terminal extension phase was performed at 72 °C for 7 min (Sambrook and Russell, 2001). The tubes were held at 4 °C until further analysis.

The PCR products were detected by electrophoresis in 0.8% agarose gels containing ethidium bromide. The gels were visualized by 300 nm long-wave ultra violet (UV) illumination in the dark room. The lengths of DNA bands were estimated by

comparing with a standard array of DNA markers (1kb plus DNA marker, Invitrogen Canada Inc., Burlington, Ontario). PCR product and primers were separated in 0.8% low melting gels containing minimum concentration of ethidium bromide. The band around 400 bps was visualized and excised under UV. The QIAEX II gene cleaning kit (Qiagen Canada Inc., Mississauga, Ontario) was used to recover the DNA fragment from the cut-out piece of agarose gel. Once the length identity of the PCR product was confirmed, it was cloned into an expression pET-32(b) vector.

C. Construction of pET-32b-PIN-b fusion protein expression vector

The purified PCR product and expression pET-32(b) vector were digested with the *Nco* I and *Xho* I using the protocols recommended by the supplier. After digestion, they were purified using the QIAEX II gene cleaning kit. The vector and PCR fragment were mixed by approximate 1:3 molar ratio and ligated by T4 DNA ligase, using the protocol from the supplier. The ligase catalyzes the ligation of double-stranded DNA between the 3'-hydroxy and the 5'-phosphate termini in the presence of adenosine triphosphate (ATP).

The standard calcium chloride protocol (Sambrook and Russell, 2001) was used for preparation and transformation of DH5α competent cells. Transformed cells were pelleted by centrifugation and resuspended in approximately 100 μL of Luria-Bertani (LB) broth and 50 μL of LB was spread on LB agar plates supplemented with 100 mg/L ampicillin. After incubating the plates overnight at 37 °C, the antibiotic

resistant colonies, if any, were picked by sterile toothpicks and grown in LB broth supplemented with 100 mg/L ampicillin overnight. These bacterial cultures were used for expression vector isolation and insert confirmation.

D. Extraction and purification of plasmid DNA

The QIAprep spin miniprep kits (Qiagen Canada Inc., Mississauga, Ontario) were used to extract and purify plasmid DNA from *E. coli* DH5α using the protocol slightly modified from the supplier. Briefly, transformed *E. coli* DH5α cells carrying the expression vector were grown overnight at 37 °C in 10 mL of LB broth supplemented with 100 mg/L ampicillin. Cells were spun down in the cold room (4 °C) to obtain the bacterial pellet. The supernatants were discarded, and the pellets were resuspended gently in 250 μL of buffer P1 and transferred into new micro centrifuge tubes. Lysis buffer P2 (250 μL) was added to the tubes. The alkaline lysis proceeded for five min before 350 μL of buffer N3 was added. The tubes were gently mixed and put in ice for 10 min. After centrifugation, the supernatants were loaded onto QIAprep spin columns. The spin columns were centrifuged 16,000 *g* at 4 °C for one min. After washing the spin columns with 500 μL of PE buffer, the plasmid DNAs were eluted with 50 μL of autoclaved H_2O.

Plasmid DNA samples were electrophoresed on 0.8% agarose gels to assess the quality and estimate the yield. Accurate quantifications were performed by using a spectrophotometer at the wavelength of 260 nm (Beckman DU300 spectrometer, Beckman Coulter Inc., Fullerton, California). The expression vector was digested

with *Nco* I and *Xho* I, and the digested vector was electrophoresed on 0.8% agarose gels to confirm that the PCR product did incorporate into the vector. The identity of insert was further confirmed by sequencing using T7 terminator primer (Biotechnology Research Institute, University of Ottawa, Ottawa, Ontario).

2.2 Expression and preparation of TRX-a-PIN-b fusion protein

A. Expression of the isotope labeled TRX-a-PIN-b fusion protein

i Establishment of bacterial culture growth

A protocol slightly modified from the standard (Sambrook and Russell, 2001) was used to express TRX-a-PIN-b fusion protein in the BL21 (DE3) host strain (EMD Biosciences Inc., San Diego, California). The BL21 (DE3) strain, which is deficient in the *OmpT* and *Lon* serine protease genes, is a commonly used *E. coli* strain for protein expression. BL21 (DE3) competent cells were transformed with PIN-b expression vector using the standard calcium chloride method. LB broth (10 mL) was inoculated with one colony of freshly transformed BL21 (DE3) in a 50 mL centrifuge tube. The culture was incubated at 37 °C on a shaker until its optical density (OD_{600}) reached 0.8. The cells were spun down at 6,000 *g* for 20 mins, resuspended in 50 mL of sterile ^{15}N-labeled M9 medium and transferred into 150 mL ^{15}N-labeled M9 medium in a 1 L flask. The flask was incubated at 37 °C with shaking. When OD_{600} of the culture reached 0.8, cells were pelleted at 6,000 *g* for 20 mins. The pellets were transferred into 1 L of ^{15}N-labeled M9 medium in a 4 L flask. The flask was incubated at 37 °C on an orbital shaker until the OD_{600} of the culture reached 0.4–0.6.

ii Induction of promoter in expression cassette

The culture was induced with isopropyl-beta-D-thiogalactoside (IPTG). The IPTG concentration, induction time and temperature had to be optimized empirically. To do this, various trial cultures were monitored. The expression level and identity of PIN-b fusion protein were monitored with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein dye-binding assay (Bradford), and Western blot, respectively. Literature reports indicated that, lower induction temperatures can lead to increased yields of soluble proteins in some cases. The increased yields of soluble proteins should be avoided due to the potential of PIN-b fusion protein to be toxic to the host cells.

iii Harvest of cells

Cells were harvested after an appropriate induction time by centrifugation at 4 °C at 6,000 *g* for 30 min. The supernatant was discarded and the bacterial pellets were stored at -80 °C until purification.

B. Protein concentration quantification

A serial dilution was made from a BSA standard (Bio-Rad Laboratories Inc., Hercules, California), which was reconstituted in phosphate buffered saline (PBS). The concentrations of BSA were adjusted to 0 (blank control), 0.05, 0.1, 0.2, 0.4, 0.6 mg/mL. The Bradford dye-binding protein assay kit (Bio-Rad Laboratories Inc., Hercules, California) was used to determine the protein concentration. After 10 min incubation of BSA standards/protein samples with Bio-Rad dye solution at 37 °C,

they were transferred into plastic micro cuvettes. The cuvettes were read by a spectrometer at the wavelength of 595 nm (Beckman DU300 spectrometer, Beckman Coulter Inc., Fullerton, California). The absorbances of the serial dilutions of BSA were plotted to determine a standard curve by the program associated with the spectrometer and/or the computer program Matlab (Mathworks Inc., Natick, Massachusetts). The protein concentrations were calculated by the program using the standard curve. Finally, the protein sample concentrations were corrected by the appropriate dilution factors (Bradford, 1976).

C. Molecular weight estimation by SDS-PAGE

The acrylamide gel for SDS-PAGE was prepared using the standard protocol (Sambrook and Russell, 2001). The resolving and stacking gels were 15% and 5% acrylamide, respectively. Concentrated SDS-PAGE loading buffer was added to the protein samples to obtain a final concentration of 1X SDS-PAGE loading buffer. After heating at 100 °C for 3 min, the samples were centrifuged and the supernatants of samples were loaded onto the wells of the acrylamide gel. The prestained SDS-PAGE molecular weight standards (Invitrogen Canada Inc., Burlington, Ontario) were used to estimate the molecular weight of the PIN-b fusion protein. The Bio-Rad Mini-Protean III cell electrophoresis system (Bio-Rad Laboratories Inc., Hercules, California) was used for the electrophoresis. The electrophoresis proceeded at the constant current of 20 mA until the blue dye of SDS-PAGE loading buffer reached the

bottom of the resolving gel. The gels were stained with Coomassie brilliant blue staining solution.

D. Identification by Western blot

Protein samples were loaded in 5% stacking gel and fractionated in 15% resolving gel at the constant current of 15 mA under denaturing condition. After electrophoresis, the gels were soaked in the Western blot transfer buffer together with filter papers and nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, California) for 30 min. Transfer of proteins from the gels to nitrocellulose membranes was performed using the semi-dry transblotter (Bio-Rad Laboratories Inc., Hercules, California) at the constant voltage of 12 V for 1 hr. Following the transfer, the gels were stained with Coomassie brilliant blue staining solution to confirm that the transfer of PIN-b fusion protein was completed.

The membranes were incubated in the Western blot blocking solution (0.25% gelatin) for 30 min at room temperature. The membranes were washed in PBST buffer. The primary polyclonal rabbit anti-PIN-b antiserum (gift from Dr. Marion, INRA, France) was diluted to 1:1,000 in the Western blot blocking solution. The membranes were probed with the primary antibody overnight in the cold room. The next day, membranes were rinsed four times in PBST. The secondary goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Sigma-Aldrich Canada Ltd., Oakville, Ontario) was diluted 1:5,000 in PBST. The membranes were treated with this secondary antibody for 1 hr at room temperature. The membranes

were rinsed extensively in PBST and incubated in the peroxidase substrate solution (Sigma-Aldrich Canada Ltd., Oakville, Ontario) until red bands appeared. The 'film development' reactions were stopped by washing the membranes in water extensively.

E. Purification of the TRX-a-PIN-b fusion protein

The protein-expression bacteria pellet harvested from 1 L M9 medium was suspended in 50 mL of soluble protein solubilization buffer. After gently stirring, the cells were lysed by sonication ten times for 18 sec pulses and pelleted again at 16,000 *g* at 4 °C for 30 min. The supernatant was discarded, and the pellet was resuspended in insoluble protein extraction buffer gently for 1 hr. The tube was centrifuged at 16,000 *g* at 4 °C for 30 min again. The pellet was discarded and the previously insoluble protein was now in the soluble supernatant fraction. After filtering the supernatant fraction through a 0.45 micrometer filter, it was ready for affinity tag purification.

The 6X His affinity tag-based purification was performed by the HIS trap kit using the protocol modified from the supplier's instruction (Amersham Biosciences Inc., San Francisco, California). Briefly, the column was washed with 5 mL of PBS supplemented with 0.02 M EDTA. This was followed by washing the column with 10 mL of H_2O before metal charging. The nickel chelating column was charged with nickel by washing the column with 2 mL of 0.1 M $NiSO_4$, and equilibrated with 10 mL of PBS. The samples were loaded and flow-through fractions were collected. After washing the column with 10 mL of PBS supplemented with 20 mM imidazole

and 8 M urea, the PIN-b fusion protein was eluted with 5 mL of PBS supplemented with 500mM imidazole and 8 M urea. The purity of PIN-b fusion protein was estimated by SDS- PAGE.

A stepwise dialysis procedure was used to incrementally reduce urea concentration. Briefly, the denatured PIN-b fusion protein was diluted to the concentration of 25 μg/mL. The diluted PIN-b fusion protein (25–50 mL in volume and 25 μg/mL in concentration) was put into the presoaked M.W. 5,000 cut-off dialysis tubing (Sigma-Aldrich Canada Ltd., Oakville, Ontario). The dialysis tubing was soaked in 2 L PBS with 8 M urea. PBS was added very slowly to dilute the urea concentration from 8 M to 6 M, 4 M, 2 M, 1 M, 0.5 M, 0.25 M, 0.125 M, 0.06 M, 0.03 M, and finally to zero molar (ten steps in total). Usually this step was performed in the cold room for 48 hr. Dialyzing away the urea in this gentle manner helped to avoid the protein aggregation. Visual inspection could detect significant precipitates (flocculation) if the urea concentration dropped too quickly.

2.3 Purification and disulfide bond facilitation of PIN-b

A. Proteolysis of TRX-a-PIN-b fusion protein

Enterokinase was added to release thioredoxin A (TRX-a) and PIN-b monomer from the fusion protein using the protocol from the supplier (New England Biolabs Inc., Beverly, Massachusetts). SDS-PAGE was used to confirm that the non-specific cleavage did not occur.

B. Disulfide bond formation facilitation

TRX-a and PIN-b mixture after proteolysis was supplemented with 1 mM phenyl methyl sulfonyl fluoride (PMSF) to inhibit the activity of protease. The protein mixture was diluted to a concentration of 0.1 mg/mL and was put into the dialysis tubing. The protein was dialyzed against protein refolding buffer in the cold room overnight with very gentle stirring. The pH and the balance of GSSG:GSH were studied to satisfy the requirements of facilitating disulfide bond formation.

C. Purification of PIN-b

The mixture of PIN-b and TRX-a monomers was put in the dialysis tubing and dialyzed against PBS. Acetic acid was added very slowly to modify the pH of PBS from 7.4 to 6.5, and this step was performed in the cold room during the time period of 24 hr. The solution was centrifuged to remove precipitates and filtered through a 0.45 micrometer filter. The solution was fractionated by HiTrap SP ion exchange column using the supplier's protocol (Amersham Biosciences Inc., San Francisco, California). Briefly, the column was washed with 5 mL of PBS supplemented with 1 M NaCl and equilibrated with 10 mL of PBS. The sample was loaded at about 1 mL/min using a syringe fitted to the column luer adaptor. The column was washed with 10 mL of PBS, and the PIN-b was eluted with 10 mL of PBS supplemented with 1 M NaCl. All fractions were collected and analyzed using SDS-PAGE.

D. Desalting and concentration of PIN-b

After verification of its identity by SDS-PAGE, the elution fraction of ion exchange

chromatography was dialyzed against 0.1 mM phosphate (pH 5.5) supplemented with 80 mM NaCl in the cold room overnight. Amicon M.W. 5,000 cut-off ultra-15 centrifugal filter units (Millipore Corporation, Billerica, Massachusetts) were used to concentrate the protein solution to the volume of 500 μL. Briefly, the Amicon unit was pre-rinsed with distilled water and loaded with PIN-b sample. The unit was centrifuged at 2,000 *g* until the retentate volume in the unit was about 500 μL.

E. Monitoring conformation change using non-reducing SDS-PAGE

Non-reducing gels were prepared using the same protocol as regular SDS-PAGE described in section 2.2C. Concentrated non-reducing SDS-PAGE loading buffer (without 2-mercaptoethanol (2ME)) was added to the protein samples to obtain a final concentration of 1X non-reducing SDS-PAGE loading buffer. After heating at 80 °C for 15 min, the samples were centrifuged and the supernatants of samples were loaded onto the wells of the acrylamide gel. Overheating the samples was avoided.
The samples treated with 2ME were not electrophoresed adjacent to those without 2ME treatment. The non-reducing gels were electrophoresed and stained using the protocol described in section 2.2C.

2.4 PIN-b bioassay

The antifungal activity of PIN-b was measured by a micro spectrophotometer as previously described (Broekaert et al., 1990; Dubreil et al., 1998). Two fungal pathogens, *Fusarium culmorum* (accession number: CCFC007853) and *Verticillium dahlia* (accession number: CCFC001921) were obtained from Canadian Collection

of Fungal Cultures, Agriculture and Agri-food Canada (Ottawa, Ontario). They were chosen as typical test organisms on the basis of their suitability as common cereal pathogens. Potato dextrose broth (PDB) (10 mL) was inoculated with fungal strains and incubated with gentle shaking at room temperature (25 °C) for 72 hr in darkness. They were diluted in the same medium to OD_{595} of 0.002 (Broekaert et al., 1990) and aliquoted into the plastic 96-well plates (30 µL per well). Different concentrations of purified and desalted PIN-b or BSA in phosphate buffer (1 mM phosphate (pH 5.5) supplemented with 80 mM NaCl) were added to each well. PDB without any protein or fungal inoculum was also aliquoted into wells as the sterile control. Each well was stirred gently with an autoclaved pipette tip in the laminar hood. The plate was covered and incubated at room temperature in the darkness for 72 hr. If the sterile control wells were contaminated as measured by OD_{595}, the 96-well plates were discarded.

The corrected absorbance (A^c) value was defined as the absorbance at 595 nm of the fungal culture measured after 72 hr minus the absorbance measured after 30 min. The A^c value at 595 nm of fungal culture without any protein (control) was defined as 100% growth. Percent growth inhibition was defined as 100 times the ratio of the A^c value of the control culture minus the A^c value of the test culture over the A^c value of the control culture (see equation below). All these absorbances were measured at 595 nm (Tecan Spectra, Seestrasse, Maennedorf, Switzerland).

$$\text{Percentage growth inhibition} = \frac{A_{control} - A_{test}}{A_{control}} * 100\%$$

Chapter III: Results

3.1 Verification of pET-PIN-b expression construct

A. Amplification of PIN-b coding sequence by PCR

The goal of this thesis is to clone, express, purify, facilitate disulfide bond formation and characterize puroindoline. The PIN-b from a diploid wheat was chosen as the model system because its grain is soft. It has the simplest genome of all wheats, and hopefully, should be transformable eventually to permit reverse/forward genetics. The coding sequence of PIN-b was PCR amplified from *Triticum monococcum* using PIN-b specific primers. The band of amplified PIN-b sequence was observed at the expected size on the agarose gel (about 400 bps) (Figure 6, Lane 4). The band was excised, cleaned, restriction digested, ligated into pET-32b, and the resulting plasmid pET-32b-PIN-b was prepared in large amount (0.84 mg) for further studies.

Rice genomic DNA was chosen as negative control because it was already known that no PIN homologous sequence has been detected in the rice genome (Tanchak et al., 1998; Gautier et al., 2000). Indeed, no PIN-b band was amplified from genomic DNAs extracted from two rice varieties (Xiushui 11 and Bin 21) (Figure 6, Lane 2 and 3).

B. Restriction analysis of the expression vector

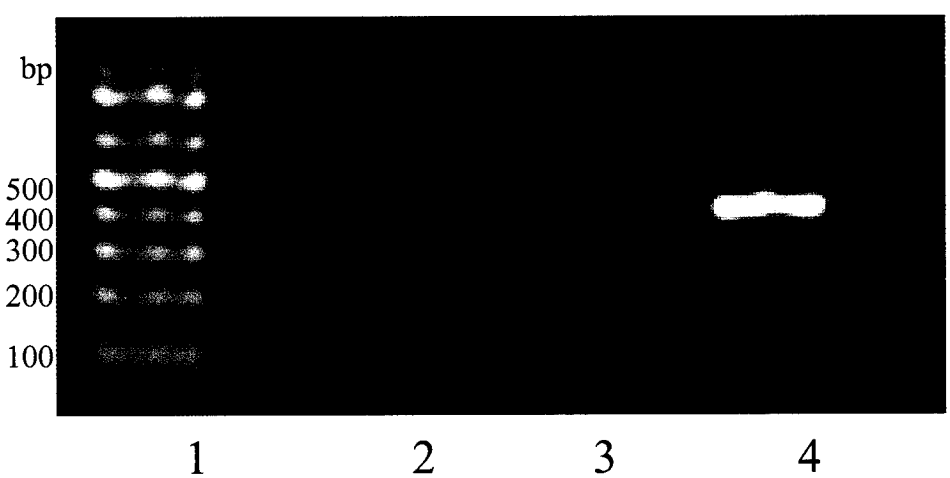
The PCR primers were designed to express PIN-b (129 amino acid residues) as a protein C-terminal to the thioredoxin (TRX-a) tag (109 amino acid residues). PIN-b protein was fused to TRX-a by a flexible linker region (50 amino acid

Figure 6 PCR of wheat PIN-b

Ethidium bromide stained agarose gel after electrophoresis showing the PCR amplification product of rice and wheat genomic DNA using PIN-b specific primer set. Forward primer was 5' cgg agc cat ggc aca ata ctc aga agt t 3' and reverse primer was 5' acg gct cga gcc agt aat agc cac t 3'.

Positions of molecular size standards (bps) are indicated to the left of the panel, corresponding to the markers in lane 1. The electrophoresis patterns of the PCR products of genomic DNA extracted from rice variety Xiushui 11, Bin-21, and wheat variety CN38852, are shown in lane 2, 3, 4, respectively.

The singular PCR product from wheat genomic DNA migrated as a 400 bp fragment in the agarose gel during the electrophoresis. As expected, the two rice control genomes yielded no PIN-b gene product.


bp
500
400
300
200
100
1
2
3
4

residues), containing the cleavable HIS•Tag (six histidine amino acid residues) and S•Tag (15 amino acid residues) sequences for purification and detection, respectively. HIS•Tag and S•Tag were removed by enterokinase digestion (S•Tag is a rapid detection system based on its interaction with RNAse S. This tag was not used in this thesis). The schematic of the construct design is shown in Figure 7A.

To confirm that the fusion protein vector was constructed as designed, DNA (500 ng) was digested with *Nco* I and *Xho* I and checked by agarose gel electrophoresis. The restriction analysis is shown in Figure 7B. The double digested vector presented the expected band pattern: the pET-32b vector backbone band (around 5.9 kbs) and the PIN-b coding sequence insert (around 400 bps).

C. Sequencing of PIN-b coding sequence

To ensure that the PCR itself and the cloning of the PCR fragment into the vector did not introduce any point mutation(s), the PIN-b expression vector was sequenced using T7 terminator primer. The sequencing report was compared to the DNA sequence deposited in the National Center for Biotechnology Information (NCBI). No mismatch was found between the reported sequence (Lillemo et al., 2002) and the sequence used in this study (Figure 8). Therefore it was felt appropriate to proceed to express this *Triticum monococcum* coding sequence in large enough quantity to permit structural elucidation.

Figure 7 Design of the pET-32b-PIN-b expression vector and its restriction analysis

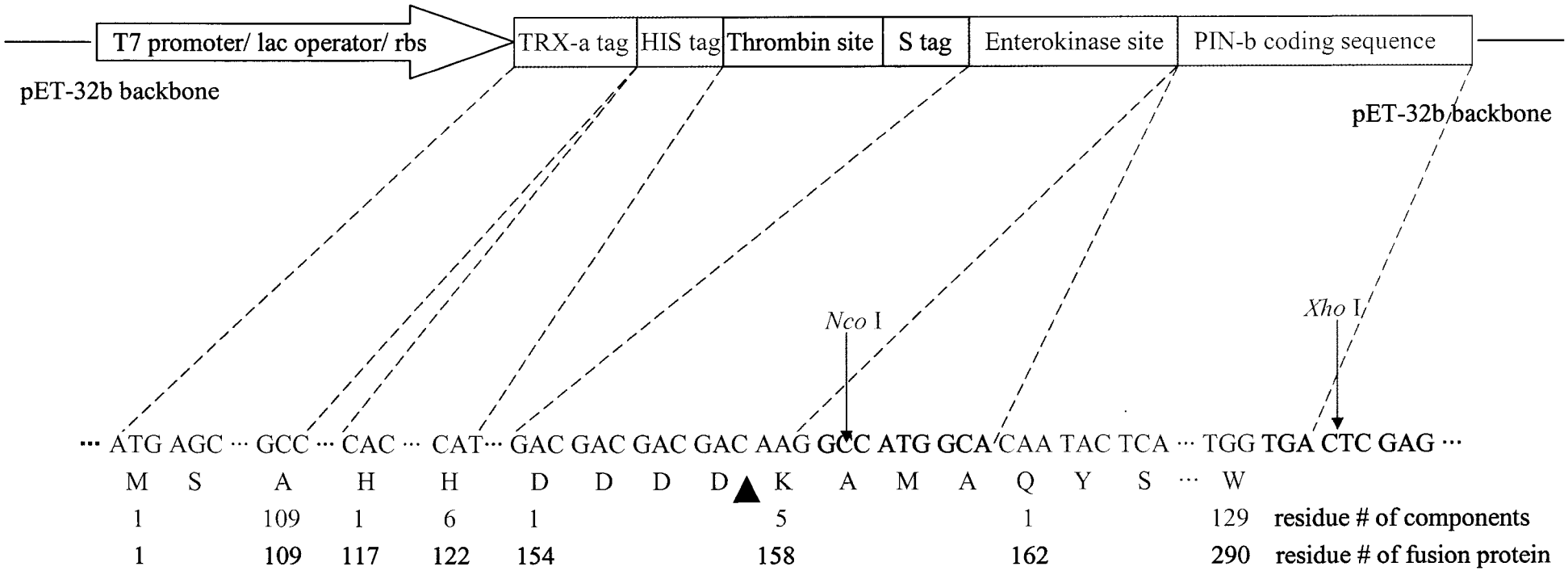
(A) The DNA/amino acid residues in blue, brown and green are the sequences for TRX-a·Tag (109 amino acid residues), HIS·Tag (six amino acid residues) and PIN-b (129 amino acid residues), respectively. The vector encoded extra amino acid residues in red are introduced during the molecular cloning process. The green arrows indicate the restriction enzyme sites exploited in the vector construction. The specific cleavage site of enterokinase is indicated by a magenta arrow. The amino acid residues in magenta are the specific recognition sequence of enterokinase.

Rbs stands for the ribosome binding sequence. The diagram is not to scale.

(B) Double restriction analysis of the pET-32b-PIN-b expression vector.

Positions of molecular size standards (bps) are indicated to the left of the panel, corresponding to the markers in lane 1 (Lambda *Hind* III) and 2 (Invitrogen 1kb plus marker), respectively. The digested vector (Lane 3) showed both the vector backbone band (5.9 kbs) and a 400 bps band at the expected size of PIN-b insert.

A


T7 promoter/ lac operator/ rbs
TRX-a tag
HIS tag
Thrombin site
S tag
Enterokinase site
PIN-b coding sequence
pET-32b backbone
pET-32b backbone
Nco I
Xho I
··· ATG AGC ··· GCC ··· CAC ··· CAT··· GAC GAC GAC GAC AAG GCC ATG GCA CAA TAC TCA ··· TGG TGA CTC GAG ···
M S A H H D D D D K A M A Q Y S ··· W
1 109 1 6 1 5 1 129 residue # of components
1 109 117 122 154 158 162 290 residue # of fusion protein

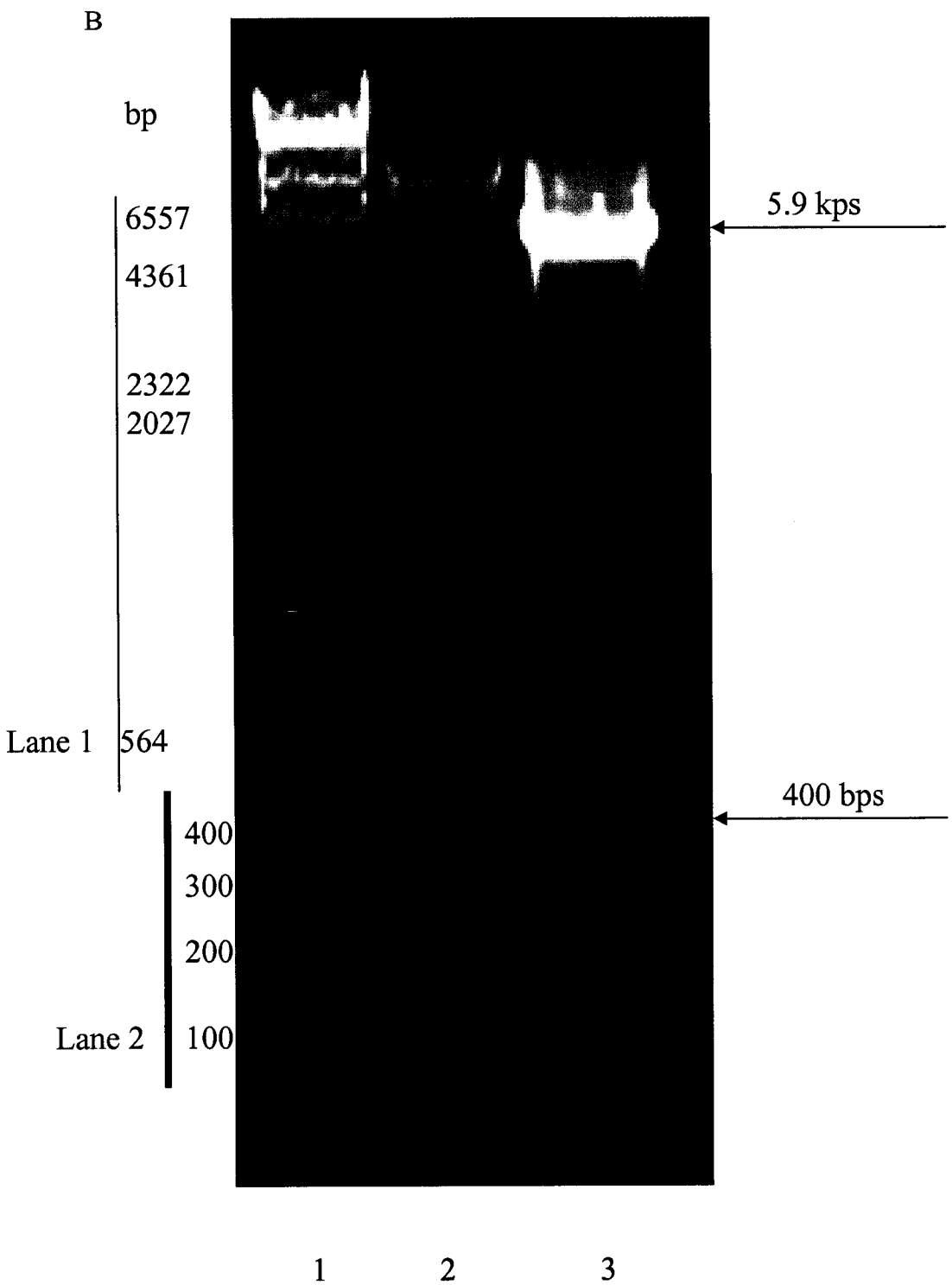

B
bp
6557
4361
2322
2027
Lane 1
564
400
300
200
Lane 2
100
5.9 kps
400 bps
1
2
3

Figure 8 PIN-b sequences alignment

Nucleotide alignment of the sequence of the pET-32b-PIN-b expression vector insert with that of PIN-b deposited in NCBI database (accession number: AJ302102; Lillemo et al., 2002). 100% identity and in-frame insertion was found in the 387 coding nucleotide alignment, indicating no mismatch or frame shift was incorporated during the PCR and vector construction. The codons coding for the ten putative cysteines are highlighted in blue type. The stop codon of the insert is capitalized. The vector derived extra coding nucleotides, introduced during the molecular cloning process, are shown in red and magenta, and their corresponding amino acid residues are shown in Figure 7A.

```
PIN-b    1 gacgacgacgacaaggccatggcacaatactcagaagttggcggctggtacaatgaagttggtgcaggaggtggttctcaacaa
Triticum                           caatactcagaagttggcggctggtacaatgaagttggtgcaggaggtggttctcaacaa
                                   ************************************************************

PIN-b    85 tgcccgctggagcggccgaagctaagctcttgcaaggattacgtgatggagcggtgtttcacaatgaaggattttccagtcacttggcccacgaaatggtggaag
Triticum 61 tgcccgctggagcggccgaagctaagctcttgcaaggattacgtgatggagcggtgtttcacaatgaaggattttccagtcacttggcccacgaaatggtggaag
            ************************************************************************************************************

PIN-b    190 ggcggttgtgagcacgaggtccgggagaagtgctgccagcagctgagccagatagcaccacagtgtcgctgcgattctatccgaggaatgatccaagg
Triticum 166 ggcggttgtgagcacgaggtccgggagaagtgctgccagcagctgagccagatagcaccacagtgtcgctgcgattctatccgaggaatgatccaagg
             ************************************************************************************************

PIN-b    288 caagctcggtggcttcttcggaatttggcgaggtgatgtattcaaacaaattcagagggcccagagcctcccctcaaagtgcaacatgggagccgac....
Triticum 264 caagctcggtggcttcttcggaatttggcgaggtgatgtattcaaacaaattcagagggcccagagcctcccctcaaagtgcaacatgggagccgac....
             ************************************************************************************************....

PIN-b    385 tgcaaattacctagtggctattactgg TGA ctcgagcaccaccac…
Triticum 361 tgcaaattacctagtggctattactgg tga
             *************************** ***
```

3.2 Expression of recombinant PIN-b fusion protein

Knowing that the PIN-b sequence was correct, the vector was then transformed into *E. coli* BL21(DE3) cells. The conditions for cell growth and gene induction in M9 minimal medium were optimized to achieve the highest expression level possible. Temperature, IPTG concentration and induction time were studied (Figure 9 and 10). Protein expression/yield was monitored by protein concentration (Bradford) of the supernatant/pellet and by SDS-PAGE. It was found that under the optimized condition (1 mM IPTG, 25 °C and 4 hr induction), 25 mg of PIN-b fusion protein (290 amino acid residues) were recovered from 1 L M9 basic medium (Figure 10). It was noteworthy that prolonged induction times and higher temperatures (1 mM IPTG, 30 °C and 6 hr induction) could still achieve similar expression levels (Figure 9 lane 9). However, the fusion protein may undergo undesirable proteolytic degradation under this condition. So the gentler condition (1 mM IPTG, 25 °C and 4 hr induction) was chosen for large-scale (2 L) production of PIN-b fusion protein.

To ensure the proper immunological identity of the major expression product, Western blot analysis was performed. The PIN-b fusion protein was recognized by polyclonal anti-PIN-b antiserum, which was provided by Dr. Marion, INRA, France (Figure 11).

Figure 9 Optimization of PIN-b expression in *E. coli* cultures

Total protein from various cultures was extracted and detected with SDS-PAGE. The acrylamide gels after the electrophoresis were stained with Coomassie brilliant blue.

Positions of protein molecular weight standards (kDa) are indicated to the left of the panel, corresponding to the markers in lane 1 (Invitrogen prestained protein Benchmark^{TM}).

pET-32b vector without the insert was induced (1 mM IPTG, 25 °C, 4 hr induction) and the total protein extract was used as a control to estimate the size of the fusion tag (the TRX-a-HIS·Tag-S·Tag fusion protein) (Lane 2). A band migrating at the size of this tag predicted from its amino acid sequence (M.W. 20,270) was observed on the gel.

The total protein of the bacteria without induction was used as induction control (Lane 3); Lanes 4, 5, 6: The total protein extracts of bacteria growing from 200 μL of culture after 2 hr, 4 hr, 6 hr induction (1 mM IPTG, 25 °C), respectively. Lane 7, 8, 9: The total protein extracts of bacteria growing in 200 μL of M9 culture after 2 hr, 4 hr, 6 hr induction (1 mM IPTG, 30 °C), respectively.

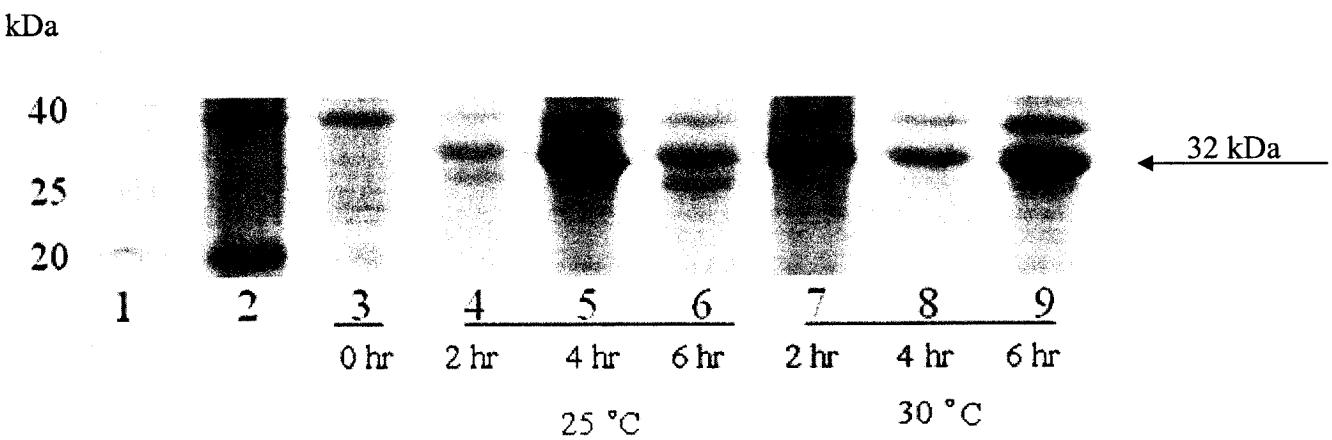

kDa
40
25
20
32 kDa
1
2
3
4
5
6
7
8
9
0 hr
2 hr
4 hr
6 hr
2 hr
4 hr
6 hr
25 °C
30 °C

Figure 10 The effects of varying IPTG concentration, induction time and temperature on TRX-a-PIN-b protein yield in M9 medium

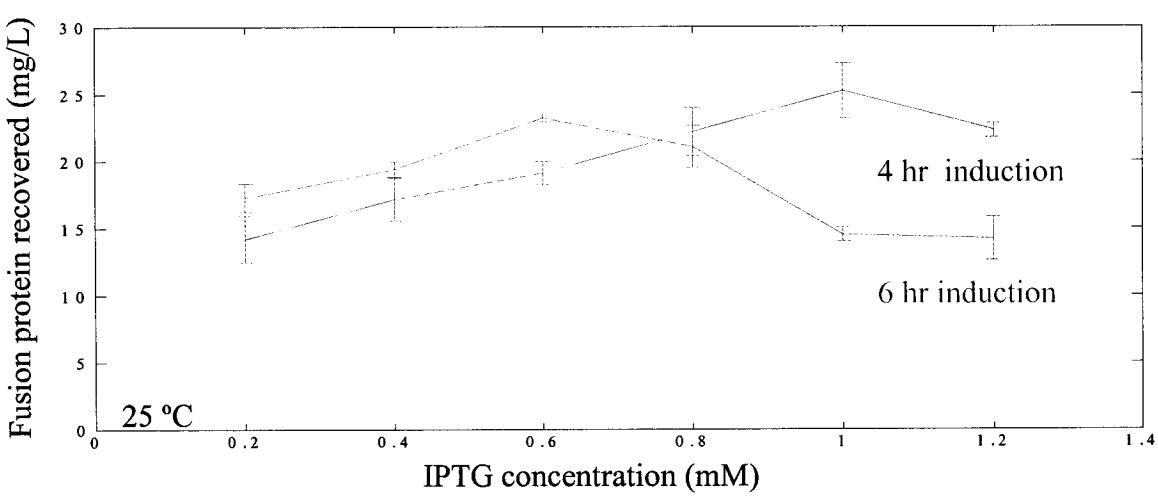
The TRX-a-PIN-b fusion protein was expressed under varying concentrations of IPTG (A), induction times (B) and temperatures (B). The error bars present the standard deviations of results from three independent experiments.

Cells were harvested after induction, and the fusion protein was purified from the total protein extracted from the cell pellets using nickel chelating column (Section 2.2E).

The purity of the purified protein was analyzed using SDS-PAGE. The gels were stained with Coomassie brilliant blue after the electrophoresis. The recovered protein was assumed to be >95% pure if a single band (32 kDa) was visualized in the gel. Induction at 25 °C and 1 mM IPTG concentration for 4 hr lead to maximal yield.

Under this tailored condition, 25 mg of PIN-b fusion protein was recovered from *E. coli* grown in 1 L M9 basic medium.

A


Fusion protein recovered (mg/L)
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
4 hr induction
6 hr induction
25 °C
IPTG concentration (mM)

B

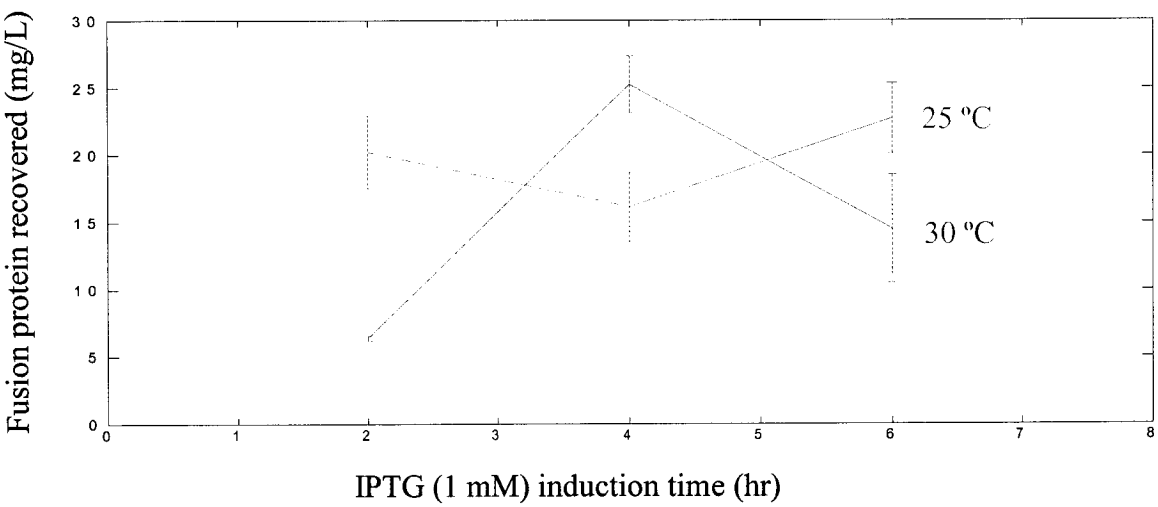
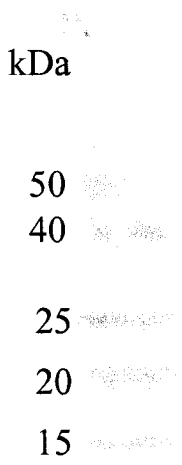

Fusion protein recovered (mg/L)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
25 °C
30 °C
IPTG (1 mM) induction time (hr)

Figure 11 Western detection of the fusion protein

Positions of protein molecular weight standards (kDa) are indicated to the left of the panel, corresponding to the markers in lane 1 (Invitrogen prestained protein Benchmark^{TM}). The total protein extract of the bacteria after 4 hr induction (1 mM IPTG, 25 °C) was loaded onto lane 2.

PIN-b fusion protein migrated in acrylamide gel (about 32 kDa) as expected and was recognized by polyclonal anti-PIN-b antiserum (Lane 2). The antiserum was a gift from Dr. D. Marion, INRA, France.


kDa
50
40
25
20
15
1
2

3.3 Purification and renaturation of PIN-b

A. Purification of PIN-b fusion protein using nickel chelating column

The workflow of PIN-b purification is illustrated in Figure 12. The fusion protein was expressed mostly in the form of inclusion bodies. The amount of PIN-b fusion protein purified from the soluble protein fraction was negligible (6%), compared to that purified from the insoluble fraction (Figure 13). For this reason, the soluble protein fraction was disregarded. PIN-b fusion protein was prepared to >95% purity by using HiTrap nickel chelating column (Figure 14).

The cells after induction were harvested, and the fusion protein was recovered from the total protein extracted from the cell pellets using a nickel chelating column. The purity of the purified protein was estimated using SDS-PAGE. The gels were stained with Coomassie brilliant blue after the electrophoresis. The recovered protein was assumed to be >95% pure if a single band (32 kDa) was visualized in the gel. Induction at 25 °C and 1 mM IPTG concentration for 4 hr lead to maximal yield. Under this tailored condition, 25 mg of PIN-b fusion protein was recovered from *E. coli* grown in 1 L M9 basic medium.

B. PIN-b protein was soluble after step-wise dialysis

The purified protein was diluted to 0.1 mg/mL to prevent undesirable precipitation. Urea was removed from the protein solution very slowly by step-wise dialysis against PBS. Otherwise PIN-b fusion protein was found to precipitate by visual inspection.

Figure 12 Workflow of PIN-b preparation

Schematic diagram illustrating the workflow of PIN-b extraction, purification, disulfide bond formation (renaturation) and concentration. A nickel chelating column was used to purify the fusion protein, and ion exchange chromatography was used to separate PIN-b from the TRX-a and the recalcitrant uncleavable fusion protein.

Extracts/Fractions labeled A through L, indicated in red type were sampled and appear in their respective SDS-PAGE lanes (Figure 13, 14, 15, 17).

DSB, disulfide bond; GSH, reduced glutathione; GSSG, oxidized glutathione; fr., fraction; PBS, phosphate buffered saline; PBSU, PBS supplemented with 8 M urea; IMZU, PBS supplemented with 500 mM imidazole and 8 M urea.

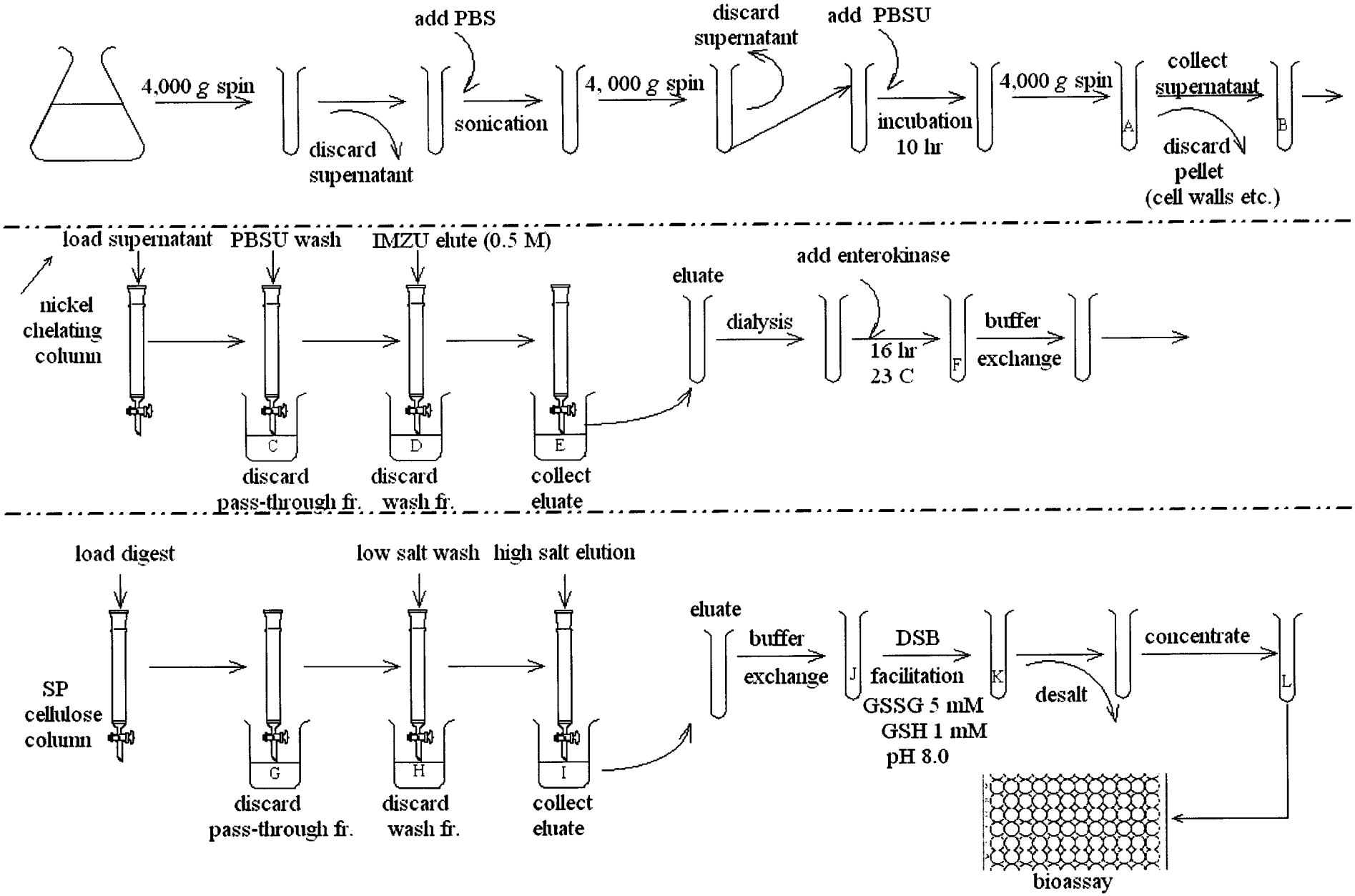

4,000 g spin
discard supernatant
add PBS
sonication
4, 000 g spin
discard supernatant
add PBSU
incubation 10 hr
4,000 g spin
A
collect supernatant
discard pellet (cell walls etc.)
B
load supernatant
PBSU wash
IMZU elute (0.5 M)
nickel chelating column
C
D
E
discard pass-through fr.
discard wash fr.
collect eluate
eluate
dialysis
add enterokinase
16 hr 23 C
F
buffer exchange
load digest
low salt wash
high salt elution
SP cellulose column
G
H
I
discard pass-through fr.
discard wash fr.
collect eluate
eluate
buffer exchange
J
DSB facilitation
GSSG 5 mM
GSH 1 mM
pH 8.0
K
desalt
concentrate
L
bioassay

Figure 13 Photograph of Coomassie brilliant blue stained SDS-PAGE gel fractionated samples, showing that TRX-a-PIN-b fusion protein was mostly expressed in the form of insoluble inclusion body

Positions of protein molecular weight standards (kDa) are indicated to the left of the panel, corresponding to the markers in lane 1. The gels were stained with Coomassie brilliant blue after electrophoresis.

The protein-expression bacteria pellet harvested from 1 L M9 medium was suspended in 50 mL of soluble protein solubilization buffer. After gently stirring, the cells were lysed by sonication and pelleted again. The total soluble protein was in the supernatant fraction (Fraction A red lettered in Figure 12), and the pellet was resuspended in 50 ml of insoluble protein solubilization buffer. After centrifugation, the pellet was discarded and the previously insoluble protein was now in the soluble supernatant fraction (Fraction B). The soluble (Fraction A) and insoluble protein extracts (Fraction B) were loaded onto Lane 2 and 3 (10 μL per well), respectively. The amount of TRX-a-PIN-b extracted from soluble protein fraction (1.5 mg per one liter of M9 culture, Fraction A) was 6% of that extracted from insoluble protein fraction (23.9 mg per liter of M9 culture, Fraction B).

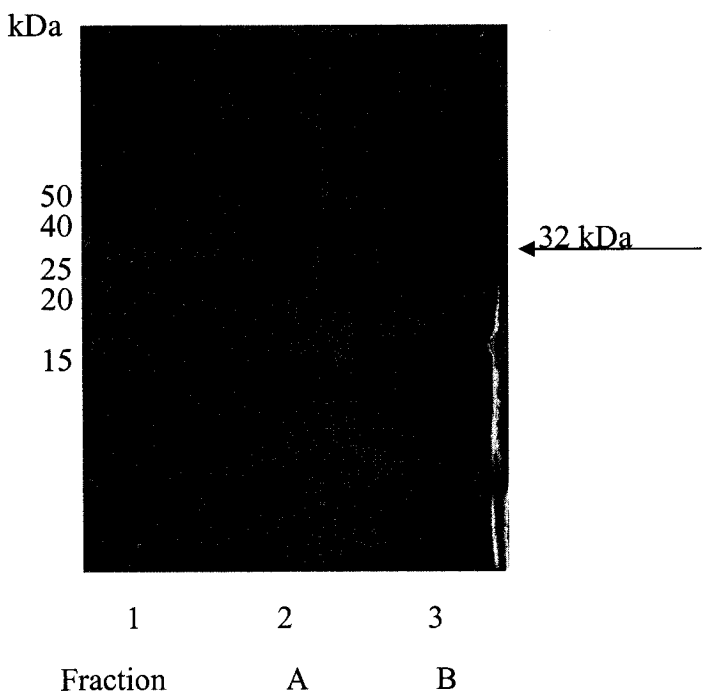
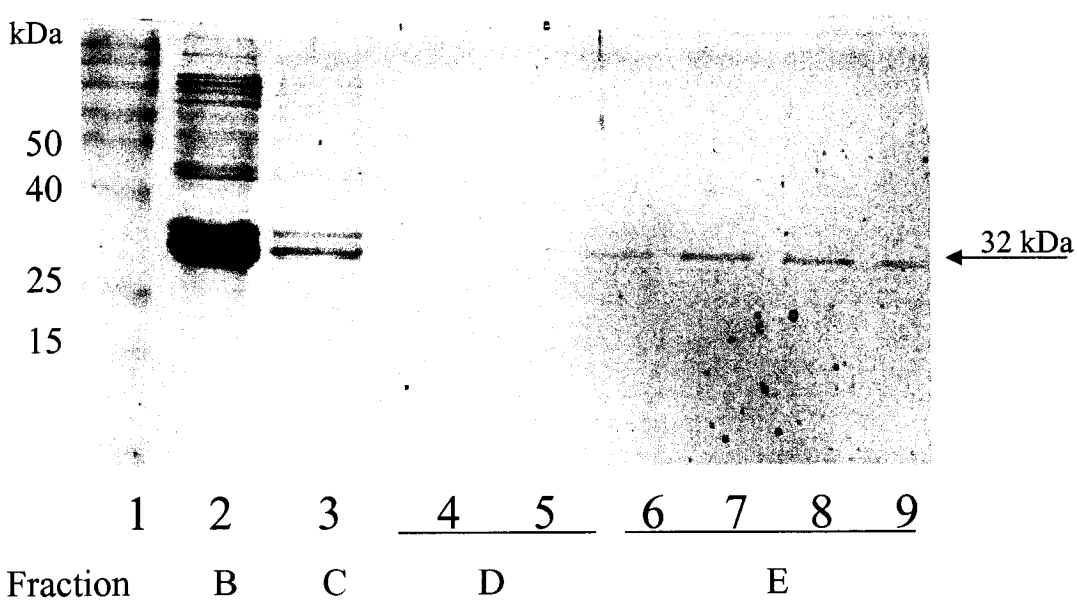

kDa
50
40
25
20
15
32 kDa
1
2
3
Fraction
A
B

Figure 14 SDS-PAGE gel of purified PIN-b fusion protein using nickel chelating column

The nickel chelating column (0.7 × 2.5 cm, flow rate 1 mL/min) from Amersham was used to purify the His-tagged PIN-b fusion protein. Different fractions after the purification were collected and analyzed using SDS-PAGE. The gels were stained with Coomassie brilliant blue after electrophoresis.

Positions of protein molecular weight standards (kDa) are indicated to the left of the panel, corresponding to the markers in lane 1. The *E. coli* pellet solubilized in 8 M urea was loaded onto lane 2. The insoluble fraction, the pass through fraction, the wash fraction (Fraction B, C, D red lettered in Figure 12, respectively), and 500 mM imidazole eluate (Fraction E) were loaded onto lanes 2, 3, 4-5, 6-9, respectively. Typically, 24 mg of the fusion protein was purified from *E. coli* grown in 1 L M9 medium.


kDa
50
40
25
15
32 kDa
1 2 3 4 5 6 7 8 9
Fraction B C D E

Protein determination by dye-binding assay (Bradford) and SDS-PAGE was used to monitor whether PIN-b was still soluble after urea was entirely removed, and to track PIN-b's electrophoretic intactness.

C. PIN-b cleavage from the TRX-a-PIN-b fusion protein

PIN-b monomer was released from the TRX-a-PIN-b fusion protein by enterokinase proteolytic cleavage. PMSF was avoided in the protease cleavage reaction since it is a strong protease inhibitor. Scanning the SDS-PAGE gels indicated that 20-35% of TRX-a-PIN-b fusion protein was not cleaved (Figure 15). Fortunately, the recalcitrant uncleavable PIN-b fusion protein was removed during the following purification process (Figure 15) and did not interfere with further experiments.

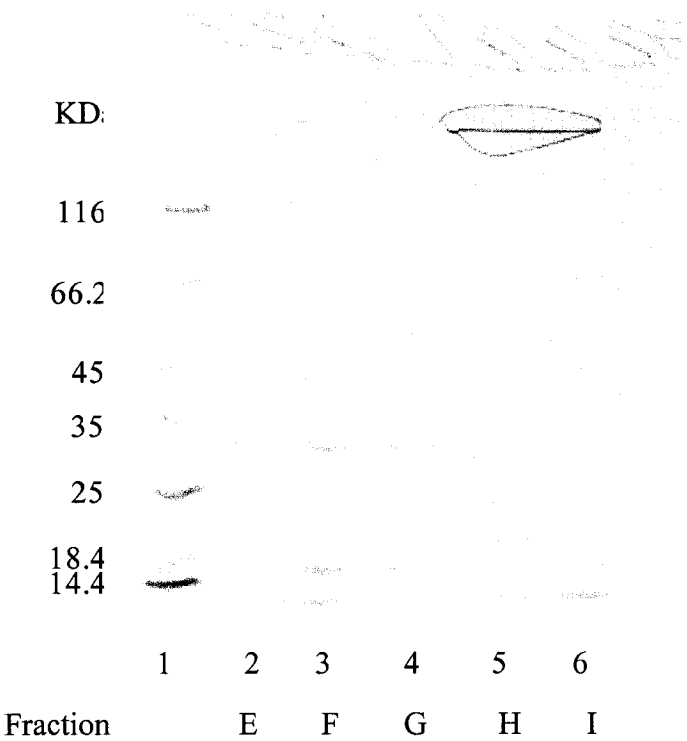
D. Purification of PIN-b

PIN-b was purified using sulfopropyl strong cation exchange chromatography. The TRX-a and the uncleavable protein were in the pass through fraction (10 mM sodium phosphate, pH 6.5), while the PIN-b was eluted in strong salt buffer (10 mM sodium phosphate, 1 M NaCl, pH 6.5). It was evident that through the ion exchange chromatography, PIN-b appeared about 95% pure, which was estimated from SDS-PAGE gel stained with Coomassie brilliant blue (Figure 15, Lane 4). The yield of the purified PIN-b (Fraction J red lettered in Figure 12) was 8.4 mg/liter of the M9 medium, and the percentage yield was 76%.

Figure 15 SDS-PAGE gel of purified PIN-b protein using sulfopropyl ion exchange chromatography

The experiments were performed four times. Results shown are from a representative experiment. Positions of protein molecular weight standards (kDa) were indicated to the left of the panel, corresponding to the markers in lane 1. The gels were stained with Coomassie brilliant blue after electrophoresis. The remarkable difference between the pI of PIN-b (pI 8.5) and TRX-a (pI 4.8) was exploited for purification.

20-35% PIN-b fusion protein was not cleaved by enterokinase (Lane 3, Fraction F red lettered in Figure 12). Steric hindrance may restrict the access of enterokinase since the fusion protein may not be folded properly. When the pH of PIN-b and TRX-a protein mixture was modified to 6.5, the PIN-b was positively charged while TRX-a was negatively charged. The samples were purified using the HiTrap SP resin (0.7 × 2.5 cm, flow rate 1 mL/min). After the purification, the different fractions were collected and examined using SDS-PAGE. TRX-a and the fusion protein did not bind to the resin and was in the pass-through fraction of the column (Lane 4, Fraction G), and no protein was in the PBS wash fraction (Lane 5, Fraction H). PIN-b bound to the negatively charged sulfopropyl resin and was eluted by high salt (Lane 6, Fraction I).


KD
116
66.2
45
35
25
18.4
14.4
1 2 3 4 5 6
Fraction E F G H I

E. PIN-b disulfide bond formation facilitation (renaturation)

The DNA sequence of the vector confirmed ten cysteine residues in PIN-b and these cysteine residues may form five disulfide bonds (Figure 8). Several recipes of varying pH and ratios of GSSG/GSH were tested to facilitate PIN-b disulfide bond formation. The fungicidal activity against *Verticillium dahliae* was chosen to evaluate the efficacies of these treatments (Figure 16A). *Verticillium dahliae* is a suitable plant pathogen model for this assay because it is sensitive to PIN-b (Dubreil et al., 1998). Disulfide bond exchange treatments proved that the tailored redox balance (PBS, pH 8.0, 1 mM GSH, 5 mM GSSG, 10 hr incubation in the cold room with very gentle shaking (Condition 2 in Figure 16A)), resulted in the most active bioassay response.

PIN-b renatured in this way demonstrated higher fungicidal activity by the comparison with the non-treated PIN-b (Figure 16B). Higher pH (pH 8.5) achieved a similar level of the enhancement (Condition 3 in Figure 16A), but this condition was poorly consistent ($n = 5$, $\sigma = 29\%$). So the lower pH (pH 8.0) was chosen for disulfide bonds facilitation. BSA used as control demonstrated a very minor effect at any concentration tested. The yield and percentage yield of the facilitated PIN-b (Fraction K red lettered in Figure 12) were 8.0 mg/liter of the M9 medium and 72%, respectively. The facilitated PIN-b demonstrated high mobility by comparison with non-treated PIN-b in the non-reducing PAGE (Figure 17).

Figure 16 Disulfide bond formation facilitation enhanced the fungicidal activity of PIN-b against *Verticillium dahliae*

(A) rtPIN-b samples were standardized by dialysis against phosphate buffer (1 mM phosphate (pH 5.5) supplemented with 80 mM NaCl) and concentration (Fraction L red lettered in Figure 12). 40 μg of rtPIN-b after various facilitation treatments or BSA (control) were added into potato dextrose broth (PDB), inoculated with *Verticillium dahliae*. rtPIN-b and *Verticillium dahliae* were incubated with gentle shaking at 25 °C for 72 hr in darkness. The fungicidal activity of rtPIN-b was measured as the percentage growth inhibition against *Verticillium dahliae* using OD_{595} measurements. The sterile control wells (PDB only without inoculum) did not show any sign of contamination using OD_{595} measurements. Average percentage growth inhibition and standard deviations stemmed from five independent replicates of the following conditions.

Condition 1: PBS, pH 7.4, 1 mM GSH, 5 mM GSSG

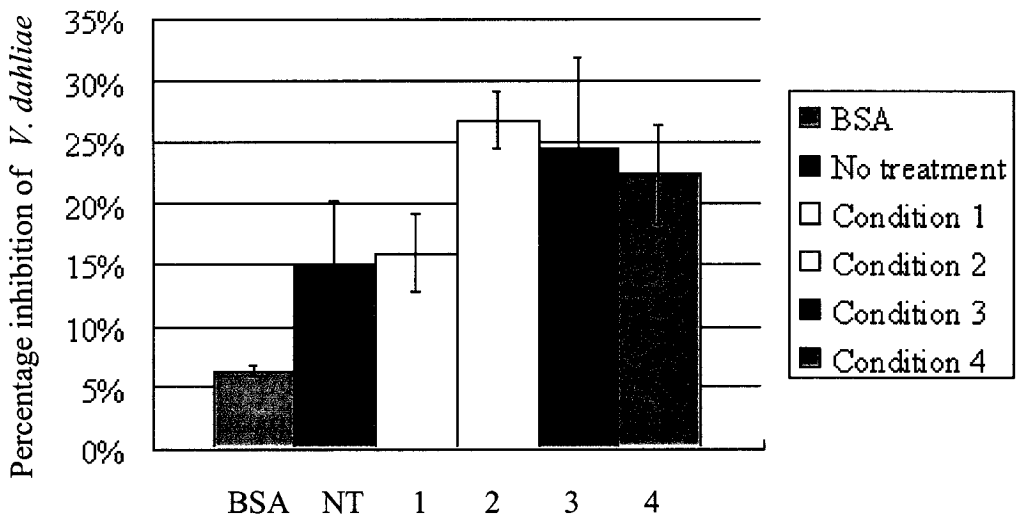
Condition 2: PBS, pH 8.0, 1 mM GSH, 5 mM GSSG

Condition 3: PBS, pH 8.5, 1 mM GSH, 5 mM GSSG

Condition 4: PBS, pH 8.0, 1 mM GSH, 2.5 mM GSSG

(B) The tailored GSH/GSSG treatment (Condition 2) enhanced the inhibition of *Verticillium dahliae*. The rtPIN-b samples without the glutathione treatment fluctuated greatly in their fungicidal activity. The results represent five independent assays.

A


Percentage inhibition of V. dahliae
35%
30%
25%
20%
15%
10%
5%
0%
BSA
NT
1
2
3
4
BSA
No treatment
Condition 1
Condition 2
Condition 3
Condition 4

B

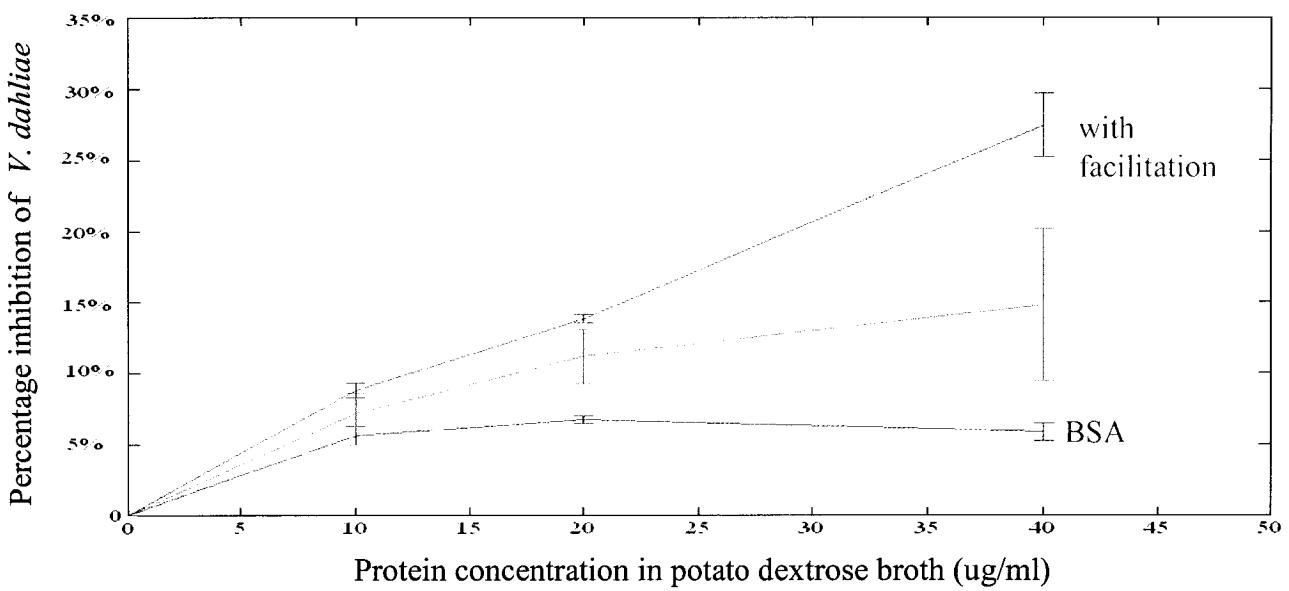
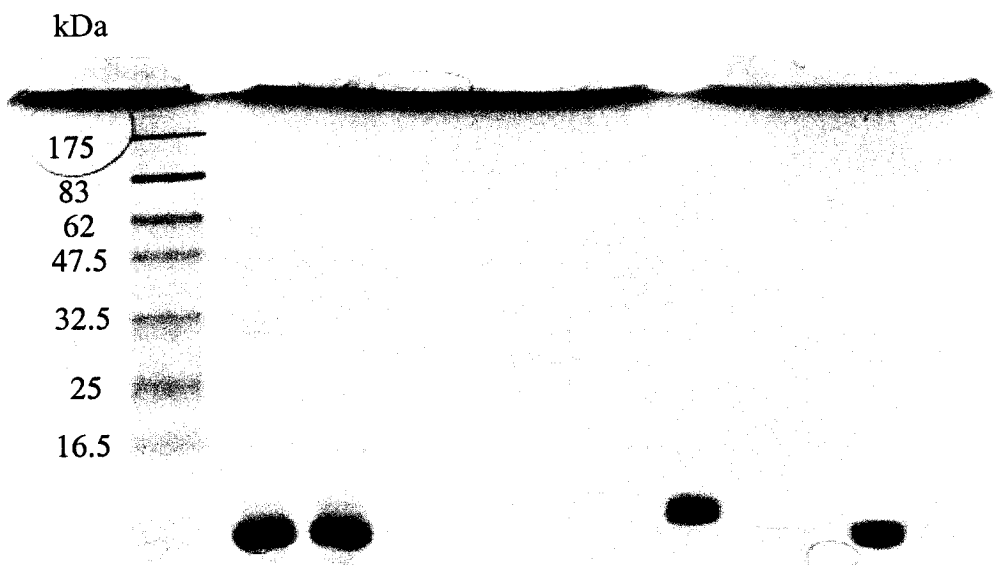

Percentage inhibition of V. dahliae
35%
30%
25%
20%
15%
10%
5%
0
0
5
10
15
20
25
30
35
40
45
50
with facilitation
BSA
Protein concentration in potato dextrose broth (ug/ml)

Figure 17 Non-reducing PAGE gel of the PIN-b before and after renaturation

Positions of protein molecular weight standards (kDa) are indicated to the left of the panel, corresponding to the markers in lane 1. The gels were stained with Coomassie brilliant blue after electrophoresis.

PIN-b samples without (Fraction J red lettered in Figure 12) or with (Fraction K) GSH/GSSG facilitation were treated with 2ME, and loaded onto Lane 2 and 3, respectively. Both of them migrated as singular 14 KDa bands in the acrylamide gel during the electrophoresis.

PIN-b without GSH/GSSG treatment (Lane 4, Fraction J) migrated as a singular band with lower electrophoretic mobility than the protein with such treatment (Lane 5, Fraction K).



	1	2	3	4	5
Fraction		J	K	J	K
facilitated		-	+	-	+
2ME treated		+	+	-	-

F. Concentration of PIN-b

PIN-b was concentrated by Centricon ultrafiltration (M.W. 5,000 cut-off) to 2.8 mg/mL (about 0.2 mM) in 20 mM phosphate, 50 mM NaCl, pH 6.5.

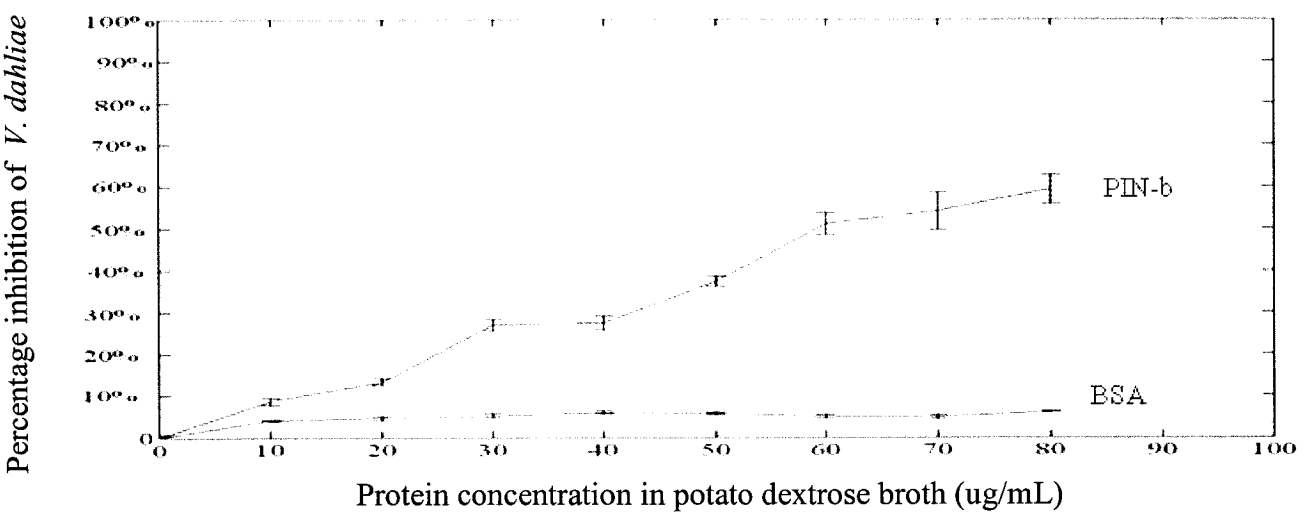
This protein sample was prepared for bioassay. An average of 7.6 mg desalted and concentrated PIN-b (Fraction L red lettered in Figure 12) was recovered from one liter of the M9 medium and the percentage yield was 71%. About one third (29%) of the protein was lost during the purification scheme.

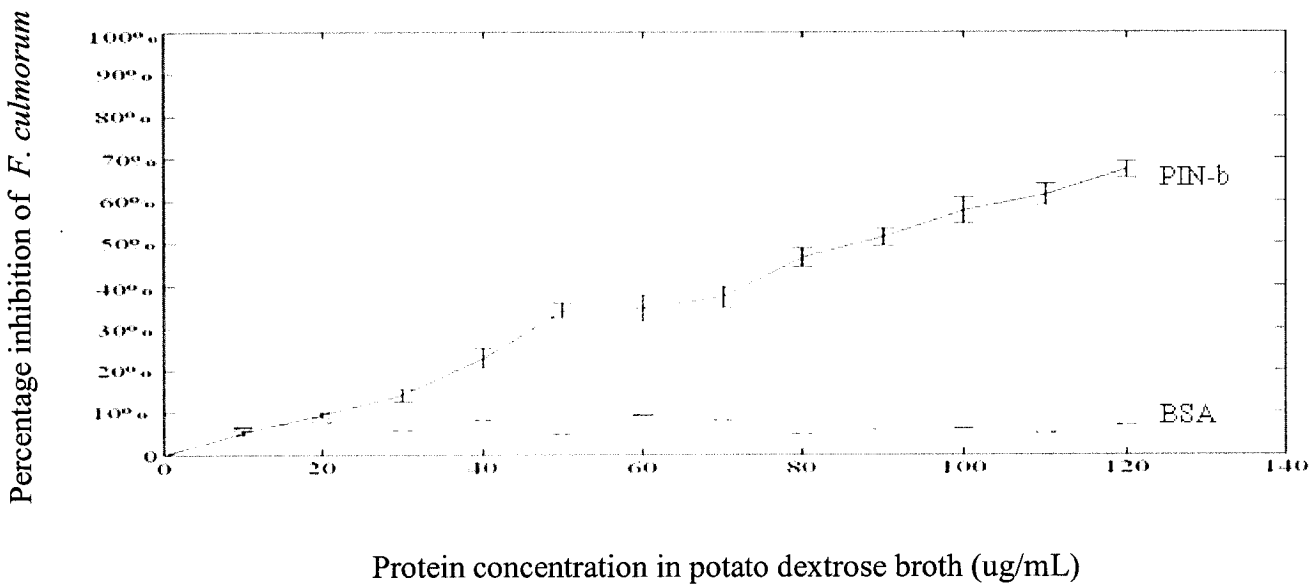
3.4 PIN-b bioassay

Verticillium dahliae and *Fusarium culmorum* are two major wheat pathogens as well as model systems for testing the efficacy of antimicrobial proteins/peptide (AMP). In vitro PIN-b demonstrated fungicidal activity against *Verticillium dahliae* and *Fusarium culmorum* (Figure 18). The concentrations inhibiting 50% fungal growth (IC_{50}) were 60 μg/mL and 90 μg /mL, respectively, while BSA did not demonstrate any considerable effect. The sterile control wells (PDB only without inoculum) were free of microbial contamination indicated by OD_{595} measurements, which suggested good sterile technique was achieved throughout the experimental process. The recombinant PIN-b purified from nickel and sulfopropyl cellulose columns was active (Fraction L red lettered in Figure 12), suggesting that this PIN-b preparation was folded properly.

Figure 18 PIN-b demonstrated its toxicity against two major plant fungal pathogens

Inhibitory curves of rtPIN-b and BSA against pathogens are shown in blue and red, respectively. In vitro rtPIN-b had earlier shown fungicidal activity against *Verticillium dahliae* (Figure 16), but the limited protein supply (due to the prohibitive price of enterokinase) did not allow a full exploration at higher concentration. The concentration of rtPIN-b that inhibited the growth of half of an inoculum of fungi was defined as IC_{50}. By adding higher concentrations of purified and desalted rtPIN-b (Fraction L red lettered in Figure 12) here in this experiment, inhibition of *Verticillium dahliae* or *Fusarium culmorum* growth greater than 50% was obtained. The IC_{50} were 60 μg/mL and 90 μg /mL, respectively. BSA used as the control did not demonstrate any considerable effect, and the sterile control wells (PDB only without inoculum) did not show any sign of microbial contamination using OD_{595} measurements. The results represented the average percentage of inhibition of three independent assays.


Percentage inhibition of V. dahliae
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0
0
10
20
30
40
50
60
70
80
90
100
PIN-b
BSA
Protein concentration in potato dextrose broth (ug/mL)


Percentage inhibition of F. culmorum
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0
0
20
40
60
80
100
120
140
PIN-b
BSA
Protein concentration in potato dextrose broth (ug/mL)

Chapter IV Discussion

4.1 Purpose of this project

One of the ultimate aims of this project was to discover the native confirmation of puroindoline-b (PIN-b), which may allow a better understanding of its function. One of the specific aims in this project was therefore to provide functional and isotope labeled PIN-b suitable for NMR structural elucidation. The main reason that PIN-b was chosen for this project is that PIN-b has stronger fungicidal activity over PIN-a (Dubreil et al., 1998). A second reason was that there are seven loss-of-function single amino acid residue mutations already characterized in PIN-b (Table 2), which are very interesting from a biophysical point of view.

The production of tens of milligrams of purified protein is a typical problem for disulfide bond rich, toxic membrane-binding proteins. PIN-b has 129 amino acid residues, of which ten are cysteines (7.2%). Since the advent of recombinant DNA technology in 1972 (Jackson et al., 1972; Berg, 1980 Nobel Prize in chemistry laureate), different expression systems have been devised and optimized to achieve the expression of toxic proteins, each with its own drawbacks. Typical problems associated with heterologous expression of this type of protein are low yield (often due to toxicity), improper post-translational modification, low stability, and partial proteolysis (Grisshammer and Tate, 1995; Miroux and Walker, 1996; Baneyx and Mujacic, 2004). Considering the prohibitive cost of the isotopic chemicals required for structure determination by solution NMR (Table 3), the most convenient and

economic expression host is *E. coli*. This thesis is focused on using the *E. coli* host system to prepare functional PIN-b.

4.2 Enhanced activity by disulfide manipulation

Native structures of proteins arise because polypeptide chains are driven to adopt structures that minimize overall Gibbs free energy, which is informed by the primary amino acid sequence (Chou, 1999). Disulfide bonds are not necessary for proper folding *per se* in many proteins, but it is generally accepted that disulfide bonds can stabilize the native confirmations of proteins. Considering the fact that most proteins need native conformations to be fully functional, it is expected that the activity of PIN-b can be enhanced since the conformation is 'reinforced' by specific disulfide formation.

Since both the structure and disulfide pattern of PIN-b are still unknown, the efficacy of glutathione and TRX-a mediated refolding cannot be evaluated in a definitive way. The study of structure-function relationships of this protein suffers from the lack of a sensitive in vitro biochemical assay (Morris, 2002). In my thesis, I estimated the structure of PIN-b by monitoring its anti-fungal activity. This assay is insensitive, and, notoriously, subject to quite a few poorly controlled variables, such as fungal strain, ionic strength of growth medium and growth measurement methods. This assay is only a rough estimation of the efficacy of GSSG/GSH treatment to facilitate disulfide bond formation.

The IC_{50} of recombinant PIN-b against *Verticillium dahliae* and *Fusarium culmorum* after optimized disulfide bond exchange as performed in this study were 60 and 90 μg/mL. These values were higher than those previously reported (Section 1.3B; Dubreil et al., 1998). One explanation is that the antifungal activity is only a rough estimation and large lab to lab variations are usual. For example, in a comparative study of the efficacies of flucytosine and amphotericin B, a 512-fold inter-laboratory variation in minimal inhibition concentration values was found when laboratories used a single published, but non-standardized methodology (Calhoun et al., 1986). An alternative explanation is that PIN-b expressed in plants undergoes other post-translational modifications (such as phosphorylation, glycosylation), hence has a more potent activity. However, there is no evidence to date of these types of modifications (Morris, 2002).

4.3 Disulfide manipulation increased the mobility of PIN-b

Although non-reducing SDS-PAGE is an insensitive and indirect method of probing intramolecular disulfide bond formation, it is possible to detect conformation changes by monitoring the protein mobility in the gel. Generally proteins with more compact conformations migrate with higher mobility in non-reducing PAGE. Glutathione redox balance treatment increased the mobility of PIN-b in non-reducing PAGE (Figure 17). The migration pattern may be interpreted as follows: the treated PIN-b may have a more compact conformation, suggesting that intramolecular disulfide bonds had been formed in the refolding process.

The mechanism of PIN-b antifungal activity is largely unknown. It is interesting that from a structural point of view, PIN-b complies with the definitions of both subcategories of linear antimicrobial protein/peptide (Table 3). PIN-b has a very unusual tryptophan-rich domain. Amongst more than a thousand identified AMPs, only indolicidin has as high a tryptophan dense region. In a study of four somewhat tryptophan-rich peptides, it was found that cationic tryptophan-rich antimicrobial peptides preferentially disrupt large unilamellar vesicles with a net negative charge. These peptides insert into the interfacial region of the phospholipid bilayer (Schibli et al., 2002). It is reasonable to suspect the same mechanism may explain the antifungal activity of PIN-b. However it was also found the tryptophan rich domain (FPVTWPTKWWKG) of PIN-b alone has too minor a bactericidal activity to be considered as a *bona fide* AMP (Jing et al., 2003).

The values of MICs of facilitated rtPIN-b against *Verticillium dahliae* and *Fusarium culmorum* were 4 μm and 6 μm, respectively. And native PIN-b purified from wheat flour had MICs against *Verticillium dahliae* and *Fusarium culmorum* of 2.6 μm and 3 μm, respectively (Dubreil et al., 1998). Although direct comparison is not available, the efficacy reported by Jing et al. (MIC>200 μm) was much lower than that found in this thesis and that previously reported (Dubreil et al., 1998). One explanation is that the overall 3D structure rather than the tryptophan-rich domain alone is involved in the antifungal mechanism. Unfortunately, sufficient information is not yet available on the regulation of PIN related genes, especially in

response to fungal infestation of grain (Morris, 2002). Although preliminary in vitro bioassay is now presented in this thesis, still it is not possible to conclude that PINs play a role in the in vivo plant defense mechanism against their pathogens in the field.

4.4 Future plans

The future goal of this project is to provide sample preparation for NMR structural determination. The single isotope labeled PIN-b has been expressed and purified. The protein after treatment facilitating disulfide bond formation demonstrated enhanced activity, suggesting that it was properly folded. This thesis provides a foundation for the future work elucidating the 3D structure of PIN-b using solution NMR.

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Appendix: preparation and composition of the media and solutions

30% Acrylamide mix
29.0% acrylamide
1.0% bis-acrylamide

Blocking buffer for Western blot
20 mM tris (pH 7.4)
0.9% NaCl
0.25% gelatin
0.1% Triton-X100
0.02% SDS

Coomassie brilliant blue staining solution
0.25% Coomassie brilliant blue R250
10% acetic acid
45% methanol
44.75% H_2O

Coomassie blue brilliant destaining solution
10% acetic acid
45% methanol
45% H_2O

CTAB solution
200 mM tris (pH 8.0)
2% CTAB
20 mM EDTA
1.4 M NaCl
1% polyvinylpyrrolidone (PVP) M.W. 40,000

DNA extraction buffer
200 mM tris (pH 8.0)
200 mM NaCl
25 mM EDTA
0.5% SDS

10X DNA loading buffer
50% glycerol
0.1 M tris (pH 8.0)
0.01 M EDTA
0.1% bromophenol blue
0.1% xylene cyanol

Enterokinase proteolysis buffer
20 mM tris (pH 8.0)
50 mM NaCl
2 mM $CaCl_2$

High salt elution buffer
20 mM sodium phosphate
1 M NaCl
pH 5.5

Insoluble protein extraction buffer
50 mM NaH_2PO_4
300 mM NaCl
20 mM imidazole
8 M urea
pH 8.0

LB agar plates
LB broth + 1% agar
LB broth
10 g/L bacto tryptone
5 g/L yeast extract
10 g/L NaCl
pH 7.0

10X M9 salt
68 g/L Na_2HPO_4
30 g/L KH_2PO_4
5 g/L NaCl

M9 medium
100 mL 10 X M9 salt
1 mL 1 M CaCl2
1 mL 1 M $MgSO_4$
1 g/L; NH_4Cl
3 g/L glucose
100 mg/L ampicillin
10 mL MEM Vitamin Solution (Invitrogen Canada Inc., Burlington, Ontario)
Water was added to bring the volume to one liter.

Nickel column elute buffer
PBS
500 mM imidazole
8 M urea

Nickel column wash buffer
PBS
20 mM imidazole
8 M urea

Non-reducing SDS-PAGE sample loading buffer
50 mM tris (pH 6.8)
2% SDS
0.1% bromophenol blue
10% glycerol

PBS
0.23 g/L NaH_2PO_4
1.15 g/L Na_2HPO_4
8.75 g/L NaCl
pH 7.4

PBST
0.23 g/L NaH_2PO_4
1.15 g/L Na_2HPO_4
8.75 g/L NaCl
0.5% Tween 20
pH 7.4

Potato dextrose agar
4 g/L potato infusion (infusion from 200 g potatoes)
20 g/L dextrose (glucose)
10 g/L agar

Potato dextrose broth
4 g/L potato infusion (infusion from 200g potatoes)
20 g/L dextrose (glucose)

Qiagen P1 buffer
50 mM tris (pH 8.0)
10 mM EDTA
10 μg/mL RNAse A

Qiagen P2 buffer
200 mM NaOH
1% SDS

Qiagen N3 buffer
3 M sodium acetate

pH 4.8

Qiagen PE buffer
50 mM tris (pH 8.0)
70% ethanol

15% Resolving gel of SDS-PAGE
2.3 mL H_2O
5.0 mL 30% acrylamide
2.5 mL 1.5 M tris (pH 8.8)
100 µL of 10% SDS
100 µL of 10% APS
4 µL TEMED

SDS-PAGE sample loading buffer
50 mM tris (pH 6.8)
2% SDS
0.1% bromophenol blue
10% glycerol
10% 2-mercaptoethanol

Soluble protein extraction buffer
0 mM NaH_2PO_4
300 mM NaCl
20 mM imidazole
pH 8.0

5% stacking gel of SDS-PAGE
3.4 mL H_2O
0.83 mL 30% acrylamide mix
0.63 mL 1.0 M tris (pH 6.8)
50 µL 10% SDS
50 µL 10% APS
5 µL TEMED
TE buffer
10 mM tris (pH 7.5)
1 mM EDTA

Transfer buffer for Western blot
48 mM tris (pH 8.3)
39 mM glycine
0.037% SDS
20% methanol

Wash buffer for Western blot
20 mM tris (pH 7.4)
0.9% NaCl
0.5% Tween 20