OPTIMIZATION OF HEAT-STABLE PROTEIN EXTRACTION IN RECALCITRANT *SPARTINA ALTERNIFLORA*

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Plant Pathology and Crop Physiology

by

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B.S. Beijing University of Chemical Technology, 2006
December 2011

ACKNOWLEDGEMENTS

I would like to thank Dr. Marc Cohn for serving as my committee chair and major professor. Thanks to committee members Dr. Zhi-Yuan Chen, Dr. Kermit Murray and Dr. John Battista, who gave me excellent and professional advice to my research project. I would like to thank Mr. Garrett Thomassie, Mr. Curt Riche and Mr. Shaun Harris, who helped me with the *Spartina alterniflora* and *Spartina spartinae* seed harvests.

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ABSTRACT

Orthodox and recalcitrant seeds exhibit differential tolerance to water loss. Recalcitrant seeds are not able to tolerate desiccation and die when dried, while the orthodox seeds can be stored dry without losing viability for years.

Spartina is a good model to study recalcitrance, because unlike most other recalcitrance studies, which contain only a recalcitrant species, this system has both recalcitrant *S. alterniflora* and orthodox species, *S. pectinata* and *S. spartinae*, as close-related physiological comparators.

Lack of protective proteins, *e.g.* late embryogenesis abundant proteins (LEAs), has been proposed to be the cause of recalcitrant seed death. A common feature of these protective proteins is their heat stability. In order to identify any heat-stable proteins that may be associated with a lack of desiccation tolerance in *S. alterniflora*, it is necessary to optimize the protocol of heat-stable protein extraction first.

Heating the protein extracts at 95^{o}C for 40 minutes and centrifuging the heated protein extracts at 20,000g and 4^{o}C for 40 minutes yield a constant protein concentration of heat-stable fractions both in the *S. alterniflora* and *S. pectinata*. Comparisons of one-dimensional SDS-PAGE gels or total protein concentration provide little information about the minimum amount of protease inhibitor needed to stop the protease activity in *Spartina* seed protein extracts. Results of the Protease Determine Quick test^{TM} protease assay indicated that 50 µl of protease inhibitor were sufficient to totally quench the protease activity in protein extracts in both *S. pectinata* and *S. alterniflora*.

To investigate an association between heat-stable fraction percentage and desiccation tolerance, heat-stable fractions of *S. alterniflora* and *S. pectinata* were

compared. There was no association between heat-stable fraction percentage and desiccation tolerance in *Spartina*. Comparative 1-DE profiles between dry *S. alterniflora* and dry *S. pectinata* did not reveal any differences. Therefore, two-dimensional gel electrophoresis, which has a much higher capability to resolve proteins, was used to investigate the differences in protein patterns between recalcitrant *S. alterniflora* and orthodox *S. pectinata*.

CHAPTER 1
INTRODUCTION

Seeds can be categorized as orthodox (desiccation-tolerant) and recalcitrant (desiccation-sensitive) (Roberts, 1973). The majority, orthodox seeds, are able to withstand extreme water loss, as much as 90-95% water removal (Vertucci and Farrant, 1995). In contrast, recalcitrant seeds do not undergo maturation drying on the mother plant; they are shed at relatively high water contents with active metabolism (Vertucci and Farrant, 1995) and fail to acquire desiccation tolerance on the mother plant, rapidly losing viability when dried and/or chilled (Pammenter *et al*., 1984).

The inability to resist desiccation and chilling challenges the storage and preservation of recalcitrant seed species. An understanding of recalcitrance will greatly contribute to improved gene banking (i.e. seed conservation) of recalcitrant seed species (Berjak and Pammenter, 2008). Studying recalcitrance is also ecologically significant. For example, *Spartina alterniflora*, which is primarily dominant in the east and gulf coast of North America, plays an important role in the estuarine ecology by accumulating sediments and providing co-habitats with other species (Walsh, 1990).

S. alterniflora, compared to other recalcitrant species, is a good model to study recalcitrance. First, *S. alterniflora* is shed dormant, and can be stored moist at 2^0C for 8-11 months (Chappell, 2008), unlike other recalcitrant species that are shed non-dormant and have a relatively short storage lifetime. Second, *S. alterniflora* seeds are relatively small and easy to handle. More importantly, either orthodox *S. pectinata* or *S. spartinae*, belonging to the same genus as *S. alterniflora*, can be used as closely-related physiological comparators to determine whether the experimental results are a common response of the genus to desiccation, which also occurs in orthodox seeds, or

recalcitrance.

The cause of the recalcitrant seed death is still a mystery. Physical damage (DNA fragmentation and seed solute leakage) and uncontrolled oxidative stress, which are considered as the cause of recalcitrant seed death in some species, were not responsible for recalcitrant *S. alterniflora* seed death; DNA fragmentation and seed solute leakage occurred during storage after seed death, and oxidative stress (lipid peroxidation and protein carbonylation) was just a response to drying in both orthodox and recalcitrant *Spartina* (Chappell, 2008). Lack of protective proteins, *e.g.* late embryogenesis abundant proteins (LEAs), may also be associated with recalcitrant seed death (Pammenter and Berjak, 1999). The abundant accumulation of these heat-stable maturation proteins coincided with the acquisition of desiccation tolerance in *Glycine max* (Blackman *et al*., 1991), and disappearance of LEA proteins was associated with loss of desiccation tolerance in *Medicago truncatula* (Boudet *et al*., 2006). A notable feature of LEAs is their heat-stability; however, there are no detailed investigations of protocols to obtain a heat stable protein fraction from seeds in the literature, *e.g.* heating temperature, duration, centrifugation speed of heated protein extract and the amount of protease inhibitor. Various investigators select a wide variety of procedures without apparent justification (see below). Therefore, optimization of the protocol of heat-stable protein extraction in *Spartina* is necessary, before the identification of any heat-stable proteins that may be associated with recalcitrant seed death. In this thesis, the following questions have been addressed: (1) what are the optimum conditions for heat-stable protein extraction, including heating temperature, duration, centrifugation speed of heated protein extract and the amount of protease inhibitor? (2) is there any association between heat-stable

protein percentages and desiccation tolerance? (3) are there any differences in the one-dimensional gel profiles of the heat-stable fraction between *S. pectinata* and *S. alterniflora*?

LITERATURE REVIEW

Oxidative Stress

Oxidative stress, attributed to an accumulation of excess reactive oxygen species (ROS), has been widely considered as a cause of the desiccation intolerance in recalcitrant seeds (Pammenter and Berjak, 1999). Oxidative stress causes damage to the membrane lipids and proteins, in conjunction with a decline of antioxidants, when seeds are desiccated (Hendry *et al*., 1992; Leprince *et al*., 1999). Increased lipid peroxidation has been associated with a loss of viability in recalcitrant and aged orthodox seeds (Wilson and MacDonald, 1986). For example, the rate of ethane evolution, an indicator of lipid oxidation (Riely *et al*., 1974), correlated with decreased viability of embryonic axes of recalcitrant *Quercus robur* L. (Finch-Savage *et al*., 1996). The TBARS (thiobarbituric acid reactive substances) assay measures malondialdehyde (MDA) (Heath and Packer, 1968), and has been widely used for the detection of seed lipid peroxidation. A sharp increase of MDA in *Theobroma cacao* coincided with a dramatic decrease in axis viability and a decline of free radical-scavenging enzymes (Li and Sun, 1999). A spike of TBARS occurred in conjunction with the loss of viability in the cotyledons, hypocotyls and root primordial of recalcitrant *Avicennia marina* seeds (Greggains *et al*., 2001). However, most recalcitrant seed studies do not include a closely-related orthodox species as a physiological 'control'. Furthermore, since the standard TBARS method (Heath and Packer, 1968) prescribes tissue homogenization at 4^{o}C, free radicals detected in an

experiment might be artifacts caused by the tissue disruption in the absence of liquid nitrogen, rather than a response to desiccation. In a comparison of orthodox *Acer platanoides* and recalcitrant *Acer pseudoplatanus*, no increases of free radicals and lipid peroxidation were observed when liquid nitrogen was used to quench the production of free radicals during homogenization (Greggains *et al*., 2000). TBARS were detected in low amounts in both dormant and non-dormant *S. alterniflora* and *S. pectinata* when seeds were freeze-clamped in liquid nitrogen, but TBARS level increased greatly when seed extraction was done at 4^{o}C (Chappell and Cohn, 2011). FOX (ferrous orange xylenol) reactive substances (hydroperoxides) were constantly low during drying of both dormant *S. alterniflora* and *S. pectinata*, but increased transiently in both non-dormant *Spartina* species (Chappell, 2008), which may result from the more active metabolism in non-dormant seeds.

Most oxidative stress studies of recalcitrant seeds have used the isolated organs (e.g. embryonic axes or embryos) instead of the whole seed (Hendry *et al*., 1992; Li and Sun, 1999; Greggains *et al*., 2000, 2001), which dry at different rates compared to the whole seed. Isolation of the axis or embryo will artificially produce free radicals (Roach *et al.*, 2008). In summary, while recalcitrant seed death has been ascribed to lipid peroxidation previously, recent evidence indicates such correlations are artifacts of the methodology or normal events that occurred during drying of both desiccation tolerant and intolerant seeds.

Physical Damage

Loss of membrane integrity caused by free radical attack could cause the recalcitrant seed death (Sun *et al*., 1994). Increased leachates that resulted from loss of

membrane integrity coincided with a decline in viability in mature embryonic axes of tea, cocoa and jackfruit (Chandel *et al*., 1995). Electrolyte leakage from recalcitrant *T. cacao* embryonic axes was stable at a low value when water content was high, but increased greatly when desiccated (Liang and Sun, 2000). Increased electrolyte leakage was coupled with decreased viability in either slowly or rapidly desiccated isolated axes of recalcitrant jackfruit seeds (Wesley-Smith *et al.,* 2001). Despite these reports, physical membrane damage may not be a cause of recalcitrant seed death: increased inorganic and organic leakage was not observed in whole seeds of *S. pectinata* or *S. alterniflora* during desiccation, but was found substantially only in both whole seeds of aged *S. alterniflora* that was stored dry at 2^{o}C, and embryos of *S. pectinata* and *S. alterniflora* isolated either before or after desiccation (Chappell, 2008). A possible explanation for the different level of leachates observed between the embryo and whole seed is that embryonic axes or cotyledons lack seed covering structures, such as the testa or pericarp, which act as significant permeability barriers that prevent small molecules from moving into or out of the tissue in both orthodox and recalcitrant seeds. However, the observed increased leachates in whole seed of aged *S. alterniflora* may rule out that explanation, because covering structures exist in both living and aged seeds. A more likely possibility is that isolation of the embryonic axis or cotyledons may induce artificial damage (Chappell, 2008; Roach *et al.*, 2008). In summary, studies in *S. alterniflora and S. pectinata* suggested that physical membrane damage is not associated with recalcitrant seed death.

Sugars

Non-reducing sugars may play an important role in conferring desiccation tolerance (Hoekstra *et al*., 2001; Berjak, 2006). The most common raffinose family

oligosaccharides [(RFOs) α-galactosyl derivatives of sucrose (Minorsky, 2003)] are abundant in most orthodox seeds, while they are absent from recalcitrant red oak (*Quercus rubra* L.) seeds (Sun *et al*., 1994).

In orthodox seeds, accumulation of sucrose and raffinose coincided with the onset of desiccation tolerance in developing soybean seeds (Blackman *et al*., 1992) and wheat embryos (Black *et al*., 1996), and a decrease in RFOs was associated with loss of desiccation tolerance in both soybean and corn axes (Koster and Leopold, 1988). RFOs accumulate in response to desiccation in the resurrection plant *Xerophyta viscosa*, which is able to tolerate and survive extreme water loss (Peters *et al*., 2007). However, the association between sugars and desiccation tolerance was challenged by studies in seeds of *Arabidopsis thaliana* and wheat embryos, which showed that accumulation of raffinose and stachyose was not associated with the acquisition of desiccation tolerance (Ooms *et al*., 1994; Black *et al*., 1999); in tomato, okra, snow pea, mung bean and cucumber seeds, loss of desiccation tolerance was not consistently associated with the decline of oligosaccharides (Lin *et al*., 1998).

The roles of sugars in protection were proposed in two hypotheses, water replacement (Crowe *et al*., 1992) and glass vitrification (Koster and Leopold, 1988). However, sugar protection is not a likely cause of difference in desiccation tolerance between orthodox and recalcitrant seeds because glass vitrification has been observed both in orthodox and recalcitrant seeds (Sun *et al*., 1994). In addition, the water content required for the occurrence of either the glass vitrification or water replacement is significantly lower than the critical water content for recalcitrant seed death (Sun *et al*., 1994).

In summary, a lack of sugar protection alone is probably not the cause of the recalcitrant seed death. However, a combination of sugars and proteins linked by hydrogen bonds was suggested to confer protection to cell membranes (Blackman *et al*., 1992; Black *et al*., 1999). Thus, a more detailed investigation of the interaction between sugars and proteins is necessary.

Late Embryogenesis Abundant Proteins

LEA proteins have been studied for over 20 years; however, their precise roles and functions in cells are still a mystery. They were first observed by their accumulation in the late stage of embryo development in cotton, *Gossypium hirsumtum* (Dure *et al.*, 1981; Galau and Dure, 1981), and named as LEAs by Galau *et al*. (1986). LEA proteins are also found in vegetative plant tissue (Hara *et al*., 2001; Mueller *et al*., 2003), pollen (Wolkers *et al*., 2001), microorganisms (Stacy and Aalen, 1998) and invertebrates (Solomon *et al*., 2000; Browne *et al*., 2002; Goyal *et al*., 2005b; Hand *et al*., 2007).

Classification of LEA proteins is a difficult task because of limited protein and cDNA data. The naming scheme of LEA proteins was initially based on LEA protein sequences from cotton, carrot, barley, rice, rape and wheat, and they have been categorized into three groups-group 1, 2 and 3 LEA proteins (Dure *et al*., 1989). Another classification was based on particular *G. hirsumtum* cDNA clones, in which D-19 for group 1, D-11 for group 2, D-11 for group 3 (Dure *et al*., 1989), D-113 for group 4, D-29 for group 5 and D-34 for group 6 (Bray, 1993). The drawback of the Dure's naming scheme is that some groups of LEA proteins, such as groups 5 and 6, were excluded from the classification (Wise, 2003). Statistically-based bioinformatics was used to re-examine the classification of the LEA proteins, and the application of peptide profile

(POPP) analysis contributed to define superfamilies within groups of LEA proteins and attempted to solve the problem caused by low sequence complexity that characterizes LEA proteins (Wise, 2003). Within the Wise (2003) naming scheme, group 1 and group 2 LEA proteins contained two and five superfamilies, respectively. The overlapping assignments within groups were not found between group 6 and LEA 14 as previous studies suggested, and LEA 5 was defined as an independent group.

A group 1 LEA protein is characterized by a 20-amino-acid sequence motif (Espelund *et al*., 1992; Hollung *et al*., 1994). Glycine is over-represented in group 1 (Espelund *et al*., 1992). Because of the abundance of glycine, group 1 LEA proteins are highly hydrophilic. Group 2 LEA proteins, also named dehydrins (Close *et al*., 1989), were characterized by the existence of conserved sequences that included K- , S- and Y-segments (Close, 1996). K-segments are highly lysine-rich motifs of 15 amino acid residues, and S- and Y- segments contain abundant serine residues and conserved sequences of seven amino acid residues in the N- terminal region, respectively (Close, 1996). Group 2 proteins also contain abundant glycine, and are highly hydrophilic. Group 3 proteins have multiple repetitions of an 11-amino-acid sequence motif (Dure *et al*., 1989) (Table 1). A more sophisticated bioinformatics tool, Pfam, refined the information about domains and families of LEA proteins based on multiple sequence alignments and hidden Markov models (Bateman *et al*., 2004) (Table 1).

Hydropathy plots (Kyte and Doolittle, 1982) of six groups of LEA proteins (used to score hydrophilicity/hydrophobicity of the peptide chain) suggested that most LEA proteins were highly hydrophilic, except for group 4 and group 5, which leads to the question ‘Can group 4 and group 5 be categorized into LEA proteins?’ From the

Table 1. LEA group motifs and corresponding Pfam families. The Pfam database can be accessed at http://pfam.sanger.ac.uk/. (Table adapted from Tunnacliffe and Wise, 2007)

Group	Motif Name	Motif Sequence	Reference	Pfam
1		GGQTRREQLGEEGYSQMGRK	Espelund *et al.*, 1992	PF00477
2	K	EKKGIMDKIKEKLPG	Close, 1996	PF00257
2	Y	(V/T)DEYGNP	Close, 1996	PF00257
2	S	Sn	Close, 1996	PF00257
3		TAQAAKEKAXE	Dure *et al.*, 1989	PF02987
4			Bray, 1993	PF03760
5			Bray, 1993	PF02987
6			Bray, 1993	PF04927
Lea5			Galau *et al.*, 1993	PF03242
Lea14			Galau *et al.*, 1993	PF03168

perspective of protein structure, people were more convinced to believe that group 4 and group 5 proteins should be excluded from the LEA proteins classification because group 5 proteins natively adopted α-helix and β-strands (Singh *et al*., 2005). Group 6 LEA was natively structured based on FoldIndex analysis (Tunnacliffe and Wise, 2007), while natively unfolded or intrinsic disorder characterized most LEA proteins, especially group 2 and group 3 LEAs. These unstructured LEA proteins lacked a folded structure but adopted a highly flexible, random-coil-like conformation in solution, and they function in an unconventional and specific way in a cell. The significance of LEA proteins being unstructured is their possible disorder-order transition under induced physiological conditions (Tompa, 2002), which were commonly observed by X-ray crystallography, far-UV circular dichroism (CD) spectroscopy, Fourier transform infrared spectroscopy and Raman optical activity measurements. The unstructured-structure transitions of LEA proteins induced by desiccation have been observed in studies of plants and seeds (Wolkers *et al*., 2001; Soulages *et al.*, 2002; Goyal *et al*., 2003; Shih *et al*., 2004; Boudet *et al*., 2006). An understanding of the disorder-order transition would help to elucidate a possible role of LEA proteins in desiccation tolerance.

The coincidence between expression of LEA mRNAs/protein and acquisition of desiccation tolerance (Dure *et al.*, 1981) inspired interests in a possible role of LEAs in desiccation tolerance. An increase of LEA mRNAs was associated with induction of desiccation stress (Hong *et al*., 1988; Close *et al*., 1989; Curry *et al*., 1991); however, LEA protein content was not evaluated in these studies. The group 1 and 5 LEA proteins were undetectable in immature embryos but gradually accumulated as embryos matured and desiccation tolerance increased in *M. truncatula* (Boudet *et al*., 2006). The

coincidence between accumulation of LEA proteins and seed/embryo maturation was also observed in castor bean (*Ricinus communis*) (Han *et al*., 1997), Arabidopsis (*Arabidopsis thaliana*) (Bies *et al*., 1998), wheat (*Triticum aestivum*) (Black *et al*., 1999), cowpea (*Vigna unguiculata*) (Ismail *et al*., 1999), almond (*Prunus amygdalus*) (Campalans *et al*., 2000), pea (*Pisum sativum*) (Grelet *et al*., 2005) and soybean (*Glycine max*) (Samarah *et al*., 2006). In addition, disappearance of LEA proteins was associated with loss of desiccation tolerance in radicles of *M. truncatula* (Boudet *et al*., 2006), and degradation of proteins of the heat-stable fraction coincided with the germination event and loss of desiccation tolerance in soybean and wheat (Blackman *et al*., 1991; Ried and Walker-Simmons, 1993).

An association between abscisic acid (ABA) and LEA proteins was reviewed by Campalans *et al*. (1999). ABA increased during embryo differentiation and reached the peak during reserve deposition, but gradually decreased after embryos began maturation drying in orthodox seeds (Still *et al*., 1994; Vertucci and Farrant, 1995). An accumulation of LEA mRNAs/proteins was also associated with an increased synthesis of endogenous ABA and increased desiccation tolerance in canola (*Brassica napus*) (Johnson-Flanagan *et al*., 1992). In addition, exogenous ABA could induce the LEA mRNAs/proteins in immature seed embryos (Galau *et al*., 1986; Hong *et al*., 1988; Johnson-Flanagan *et al*., 1992; Campalans *et al*., 2000; Borovskii *et al*., 2002; Cuming *et al*., 2007), and LEA mRNAs/protein could be re-induced by exogenous ABA in desiccation-intolerant germinating seedlings of orthodox species (Mundy and Chua, 1988; Ried and Walker-Simmons, 1993; Moons *et al*., 1995). Despite this evidence supporting an association between LEA proteins and ABA content, regulation of the expression of *lea* genes was

not identical with the pathway of ABA regulation (Pammenter and Berjack, 1999), which suggested that expression of *lea* genes was independently present in seeds, while ABA directly played an important role in desiccation tolerance. In addition, while a low [ABA] was found in recalcitrant *T. cacao* (Pence, 1991) and ten tropical-wetland recalcitrant species (Farrant *et al*., 1996), high [ABA] were observed in recalcitrant *Castanospermum austral*, *Camellia sinensis* and *Castanae sativa* (Farrant *et al*., 1996). Thus, while increased ABA content may be generally correlated with desiccation tolerance, it is not the only factor determining recalcitrance.

The relationship between an absence of LEA proteins and recalcitrance is not conclusive. On one hand, new synthesis of heat-stable maturation proteins was not detected in late stage embryogenesis in recalcitrant *Avicennia marina* (Farrant *et al*., 1992), and dehydrin-like proteins were absent from ten tropical-wetland recalcitrant species (Farrant *et al*., 1996). Dehydrins were not detected in recalcitrant *A. pseudoplatanus* (sycamore), but were present in orthodox *A. platanoides* (Norway maple) (Greggains *et al*., 2000). On the other hand, dehydrin-like proteins were detected in both orthodox and recalcitrant *Acer* species (Gee *et al*., 1994), recalcitrant seeds of the temperate species *Zizania palustris* (Bradford and Chandler, 1992; Still *et al*., 1994), recalcitrant *Quercus robur*, *Castanea sativa*, *A. pseudoplatanus* species (Finch-Savage *et al*., 1994) and warm and cold temperate recalcitrant species (Farrant *et al*., 1996). Thus, recalcitrance may not simply result from an absence of LEA proteins. However, there are caveats to this controversy that need to be addressed: (1) the specificity of antibodies of LEA proteins was questionable, in that LEA proteins were detected by Greggains *et al*. (2000) but were absent (Finch-Savage *et al*., 1994; Farrant *et al*., 1996) in the same

recalcitrant *A. pseudoplatanus* species, even though radicles and cotyledons of *A. pesudoplatanus* were used in Greggains *et al*. (2000) while axes were used in Farrant *et al*. (1996); (2) even if LEA proteins are present in recalcitrant species, a difference in the concentration and type of LEA proteins may affect the desiccation tolerance. Immunocytochemical studies revealed that group 1 and group 4 LEA proteins accumulated to 13.6 nmol/embryo and 17.0 nmol/embryo, respectively, in cotton (Roberts *et al*., 1993). Group 2 and 3 LEAs were more likely to be associated with desiccation- and chilling- tolerance, while type 1 LEAs were mostly coupled with chilling- tolerance (Tunnacliffe and Wise, 2007). Furthermore, LEAs/maturation proteins might play an important role in desiccation tolerance together with non-reducing sugars (Blackman *et al*., 1992; Walters *et al*., 1997); so a combination of LEA proteins and sugars may play a more important role in seed desiccation tolerance than LEAs alone.

After the hypothesis that LEA proteins might play an important role in conferring desiccation tolerance was introduced, the next question was 'how do LEA proteins protect cells from desiccation damage?' First, LEA proteins were suggested to prevent sensitive Krebs cycle enzymes from aggregation when desiccated, e.g. malate dehydrogenase, citrate synthase and fumarase. Group 3 LEAs alone failed to confer protection to these enzymes, but were able to prevent lactate dehydrogenase from aggregation together with trehalose under heat- and desiccation-stress *in vitro* (Goyal *et al*., 2005a). A similar protection of lactate dehydrogenase by LEAs was observed by Reyes *et al*. (2005) *in vitro*. Adding enough amounts of a group 3 LEA protein isolated from pea seed mitochondria prevented fumarase from denaturation during desiccation *in vitro* (Grelet *et al*., 2005). Besides *in vitro* evidence of protection by LEAs, group 3 LEA

proteins from desiccation-tolerant *Aphelenchus avenae* (a nematode species) prevented proteins from aggregation both *in vitro* and *in vivo* (Chakrabortee *et al*., 2007). However, the amount of LEAs added in these experiments was far beyond the physiological concentration of LEAs in a cell (Chakrabortee *et al*., 2007). A possible protection for desiccation-sensitive enzymes by LEAs is reminiscent of the function of chaperones, which can assist the re-folding/unfolding and the re-assembly/disassembly of proteins when the proteins function abnormally (Ellis and van der Vies, 1991; Hendrick and Hartl, 1993). Especially, superfamilies of LEAs, such as groups 1b, 2b, 3a and 6, were suggested to function similarly as chaperones by POPP analysis (Wise and Tunnacliffe, 2004). The shared peptide profiles between LEAs and heat-shock proteins/chaperones may facilitate the understanding of the function of LEAs, but at the same time may complicate the situation because it is not known whether LEAs function independently or if they are actually a group of heat-shock proteins (Mtwisha *et al*., 1998).

In addition to a function as chaperones, LEA proteins were suggested to play a role in ion binding in the cell (Wise and Tunnacliffe, 2004). Phosphorylation of the dehydrin, ERD14 (shown as shifts in SDS-PAGE), was associated with calcium binding that was confirmed by MALDI-TOF mass spectrometry, and the S segment of dehydrin (poly-serine) was suggested to provide the sites for calcium binding (Alsheikh *et al*., 2003; 2005); but whether the ion binding ability of the dehydrin acts as an ion buffer or acts as an ion dependent chaperone protection was not demonstrated. A further study revealed that two other dehydrins, COR47 and ERD10, exhibited phosphorylation-dependent calcium binding, but dehydrins, RAB18 and XERO2, did not show calcium binding activity (Alsheikh *et al*., 2005). The different calcium binding activities of these

proteins may be due to different motifs of their respective superfamilies (Alsheikh *et al*., 2005). COR47, ERD10 and ERD14 are acidic dehydrins, but RAB18 and XERO2 are grouped into basic/neutral dehydrins. Interestingly, zinc and iron have a higher binding affinity to dehydrins compared to calcium (Alsheikh *et al*., 2005; Hara *et al*., 2005). The high binding affinity of dehydrins to metal ions suggested that dehydrins may indirectly function as antioxidants (Tunnacliffe and Wise, 2007). Catalytic iron could be released when cells were desiccated, and would be able to generate reactive oxygen species (ROS) (Iturbe-Ormaetxe *et al*., 1998). Dehydrins of *Citrus unshiu* showed scavenging activity for hydroxyl radicals and prevented peroxidation of liposomes from soybean *in vitro* (Hara *et al*., 2003). Thus, no difference in oxidative stress between *S. pectinata* and *S. alterniflora* (Chappell, 2008) may be explained by a possible presence of dehydrins in both *S. pectinata* and *S. alterniflora*. Since LEAs were also found in recalcitrant species (Bradford and Chandler, 1992; Finch-Savage *et al*., 1994; Still *et al*., 1994; Farrant *et al*., 1996), the LEAs may inhibit the generation of ROS and keep the oxidative stress at a low level that could not be detected by chemical reagents.

A combination of LEAs and non-reducing sugars may play a more significant role in maintaining membrane integrity than non-reducing sugars alone. A combination of dehydrin from pollen and desiccated sucrose had higher glass transition temperature and strength of hydrogen bonding than desiccated sucrose alone *in vitro* (Wolkers *et al*., 2001). LEA proteins may interact with other macromolecules, and the accumulation of chaperone-like LEA proteins near the membrane surface may stabilize the membrane integrity (Hoekstra *et al*., 2001). However, evidence also indicated that accumulation of dehydrins did not coincide with occurrence of raffinose throughout induction of

desiccation tolerance in immature wheat embryos (Black *et al*., 1999).

Results in expression of *HAV1*, a group 3 *LEA*, in transgenic rice (*Oryza sativa*) directly provided the evidence of a role of LEA proteins in conferring vegetative tissue tolerance to water deficit and salt stress (Xu *et al*., 1996). The function of LEAs in drought tolerance was confirmed in another study in which transgenic Basmati rice with *HVA1* showed higher drought- and salt- tolerance and lower solute leakage (Rohila *et al*., 2002). Under a drought stress cycle, transgenic rice (*Oryza sativa*) that expressed exogenous *HAV1* had enhanced the drought tolerance indicated by lower solute leakage (Babu *et al*., 2004).

The optimization steps needed to obtain a heat-stable fraction are very important, since many previous papers report a wide range of extraction and processing conditions, but do support the stated protocols experimentally. The heat-stable fraction was selected for proteomic evaluation for my research for several reasons. (1) A number of putative protective proteins against desiccation (e.g. LEAs, heat-shock protein, superoxide dismutase) have elevated heat-stability (Nice *et al.*, 1994; Tunnacliffe and Wise, 2007).(2) Increased chances of macromolecular interaction under water stress cause protein denaturation (Hoekstra *et al.,* 2001), which appears similar to protein folding and denaturation by heating stress; therefore, it is generally assumed that the heat stable proteins *in vitro* are also able to resist water stress *in vivo*.(3) The heating of the total soluble protein solution can serve as a protein pre-fractionation step, giving a simpler proteome to work with. Therefore, optimization of heat-stable protein extraction was studied as a prelude to evaluation of the role of such proteins in seed desiccation tolerance.

CHAPTER 2
OPTIMIZATION OF HEAT-STABLE PROTEIN EXTRACTION IN *S. PECTINATA* AND *S. ALTERNIFLORA*

LITERATURE REVIEW

Heat-stable Proteins

One feature of late embryogenesis abundant (LEA) proteins is that they are highly hydrophilic and resist heat precipitation from a solution (Pammenter and Berjak, 1999; Tunnacliffe and Wise, 2007). Heating treatment is a critical step in the isolation of heat-stable proteins. Heating temperature used in various investigations (Table 2.1) ranged from 70^0C to 100^0C. But the optimization of heating temperature and duration for isolation of heat-stable protein has not been published. In addition, the optimum heating temperature or duration in *S. alterniflora*, *S. pectinata* and *S. spartinae* seeds may not be the same as in other systems. The purpose of this experiment was to identify a proper heating temperature and duration that yield a heat-stable fraction whose concentration was constant after heating. However, the heat-resistance of different LEA protein classes and the boundary between LEA proteins and other heat-stable proteins is not clear. For example, can one conclude that proteins are not heat-stable proteins because they are able to resist the heat denaturing at 80^oC, but fail at 90^oC? It may be possible that the desired LEA proteins could only survive at 80^oC but are denatured at 90^oC. It is even possible that some LEAs proteins are heating unstable, taking the fact that group 4 and 5 LEAs are hydrophobic proteins (Tunnacliffe and Wise, 2007). In this case, the desired proteins will be precipitated. Therefore, in order to avoid discarding any possible useful protein, a comparison of protein profiles between the total fraction and heat-stable fraction is necessary.

Table 2.1. Different heating temperatures and heating durations, number of papers using each temperature and heating duration, and references for isolation of a heat-stable protein fraction from plant tissues.

Temperature and Heating duration	**# papers**	**Reference**
No Heating Treatment	1	Roberts *et al*., 1993
70°C for 10 min	4	Ismail *et al*., 1999; Korol and Klein, 2002; Jayaprakash *et al*., 1998; Ried and Walker-Simmons, 1993
75°C for 10 min	1	Azarkovich and Gumilevskaya, 2006
80°C for 10 min	5	Blackman *et al*., 1991, 1992; Farrant *et al*., 1992; Garello *et al*., 2000; Mtwisha *et al*., 1998
80°C for 20 min	1	Manfre *et al*., 2006
80°C for 30 min	2	Mtwisha *et al*., 1998; Wolkers *et al*., 2001
85°C for 10 min	5	Hara *et al*., 2001, 2003, 2004, 2005; Panza *et al*., 2007
85°C for 20 min	2	Alsheikh, *et al*., 2003, 2005
90°C for 10 min	1	Capron *et al*., 2000
95°C for 10 min	2	Boudet *et al*., 2006; Grelet *et al*., 2005
100°C for 10 min	13	Aarati *et al*., 2003; Bettey *et al*., 1998; Bian *et al*., 2002; Bradford and Chandler, 1992; Close *et al*., 1989; Greggains *et al*., 2000; Han *et al*., 1997; Hara *et al*., 2001; Jacobsen and Shaw, 1989; Pelah *et al*., 1995; Soulages *et al*., 2002, 2003; Still *et al*., 1994
100°C for 15 min	2	Kikawada *et a*l., 2006; Samarah *et al*., 2006
100°C for 20 min	1	Borovskii *et al*., 2002; Houde *et al*., 1992
100°C for 10 or 20 or 30 min	1	NDong *et al*., 2002

Most proteins will be denatured when heated in solution. The three-dimensional structure of proteins will break, and the structure of the protein/peptide begins to unravel when the protein solutions are heated. The hydrophobic parts of the protein will come closer to form the aggregates (Tanford, 1968). While cold precipitation is a common way to speed up formation of precipitates of denatured proteins, little information about the optimum duration for cold precipitation of heat denatured seed proteins has been reported in the literature (Table 2.2). Therefore, it is necessary to check the optimum duration for cold precipitation of heat-unstable proteins in the *S. alterniflora* and *S. pectinata* seeds.

After heating and ice precipitation, the denatured proteins are removed from the supernatant by pelleting with centrifugation. Different centrifugation speeds and durations (Table 2.3) have been used previously. Thus, experiments were performed to investigate the effect of different centrifugation speeds and duration on the precipitation of heat-unstable proteins in the *S. pectinata* and *S. alterniflora* seeds. Because a tested centrifugation speed was limited by both the centrifuge instrument and maximum speed that centrifuge tubes could withstand, the speeds of 14,000 g, 16,000 g, 18,000 g, or 20,000g for 10, 20, 30, 40 or 60 minutes have been evaluated.

Percent Heat-stable Protein and Desiccation Tolerance

The presence of heat-stable LEA proteins may be associated with the acquisition of desiccation tolerance in orthodox seeds (Grelet *et al*., 2005), and LEA proteins are absent in some recalcitrant seed species (Farrant *et al*., 1996; Greggains *et al*., 2000). The comparisons of fraction of heat-stable proteins may provide indication as to the relationship between LEAs and desiccation tolerance, because one of characteristics of LEA proteins is heat-stability (Pammenter and Berjak, 1999). However, the heat-stable

Table 2.2. Different duration of cold incubations, the number of papers using each duration, and references.

Duration of Cold Incubation	# of Papers	References
-50^{o}C for 5 minutes	2	Alsheikh *et al*., 2003, 2005
Ice for 4 minutes	1	Samarah *et al*., 2006
Ice for 5 minutes	1	Pelah *et al*., 1995
Ice for 15 minutes	3	Boudet *et al*., 2006; Bradford and Chandler, 1992; Close *et al*., 1989

Table 2.3. Different centrifugation speeds and durations, number of papers, and references.

Centrifugation Speed, Duration and Temperature	# of Papers	Reference
10,000g for 5 min	1	Bradford and Chandler, 1992
10,000g for 10 min	1	Pelah *et al*., 1995
10,000g for 10 min at 4°C	2	Aarati *et al*., 2003; Panza *et al*., 2007
10,000g for 15 min at 4°C	1	Hara *et al*., 2001
11,000g for 15 min	1	Kikawada *et a*l., 2006
12,000g for 10 min	2	Jayaprakash *et al*., 1998; NDong *et al*., 2002
12,000g for 10 min at 4°C	5	Bettey *et al*., 1998; Hara *et al*., 2001, 2003, 2004, 2005
13,000g for 10 min 4°C	1	Boudet *et al*., 2006
13,000rpm for 15 min at 4°C	1	Ried and Walker-Simmons, 1993
14,000g	1	Greggains *et al*., 2000
14,000g for 15 min at 4°C	2	Grelet *et al*., 2005; Han *et al*., 1997
15,000g for 10 min	1	Wolkers *et al*., 2001
15,000g for 10 min at 4°C	1	Mtwisha *et al*., 1998
15,000g for 20 min	1	Houde *et al*., 1992
16,000g for 10 min	2	Blackman *et al*., 1991, 1992
26,500g for 20 min	2	Soulages *et al*., 2002, 2003
30,000g for 60 min at 4°C	1	Ismail *et al*., 1999
35,000 for 10 min at 5°C	1	Capron *et al*., 2000
100,000g for 60 min at 4°C	2	Alsheikh *et al*., 2003, 2005

fraction in seeds of *Spartina* cannot be assumed to contain only LEA proteins, since proteomic evidence has not been obtained to date. References indicated 19% of total protein was heat-stable (HS) in desiccation sensitive *Medicago truncatula* radicles (Boudet *et al*., 2006) and 25-30% in *Glycine max* axes during development and germination (Blackman *et al*., 1991). HS protein fractions among *S. alterniflora*, *S. pectinata*, *G.max* and other species will be compared to examine whether desiccation tolerance is related with percent HS protein fractions.

Protease Inhibitor in Protein Extracts

Proteases, which hydrolyze the peptide bonds in proteins, lead to decreased yields of proteins during protein extraction (Barrett *et al.*, 2003; Kovacs *et al.*, 2008). Proteases are classified into six groups that include serine, threonine, cysteine, aspartic acid, metallo- and glutamic acid proteases (Barrett *et al.*, 2003). Protease inhibitors are chemicals that are able to inhibit the hydrolysis of peptides by these proteases. Since a small amount of protease is sufficient to degrade the proteins (White *et al*., 1993), leading to an erroneous interpretation of proteomics data, it is very important to know that sufficient protease inhibitor is added to completely stop the protease activities. Thus, the minimum amount of protease inhibitor needed to completely stop protease activities was tested in *Spartina*. Since 50 µl of protease inhibitor is recommended for inhibition of proteases in 5 ml of extraction buffer (Sigma-Aldrich), protease inhibitor (#P9599, Sigma Aldrich, Sigma Chemical Company, Saint Louis, MD, USA) from 0 µl to 50 µl was added into the extraction buffer. Comparisons of protein extract concentration and one-dimensional SDS-PAGE profiles were used to evaluate the minimum amount of protease inhibitor to completely stop protease activity in protein extracts.

A sensitive measure of proteolysis was also used to confirm the effectiveness of protease inhibitor activity. The PDQ (Protease Determine Quick Test)^{TM} protease assay (AthenaES, Environmental Science Inc., Baltimore, USA) was chosen to investigate the inhibition effect of protease inhibitor on protease activity of extracts in the *S. alterniflora* and *S. pectinata* seeds. The PDQ protease assay was used because of its ease as a colorimetric assay. The substrate, which responds to various proteases including serine, metallo, aspartate and cysteine proteases, is a cross-linked matrix containing protein substrate and a dye-protein conjugate. Protease activity is detected spectrophotometrically with increasing absorbance proportional to increasing enzyme activity.

METHODS

Seed Materials

S. alterniflora seeds were harvested in November, 2006-2008 from marshes of Port Fourchon, Louisiana. Seeds were collected by hand shattering. Seeds were immediately sealed in plastic zipper bags when harvested and put in a cool place after harvest. After transportation to laboratory, ten gram aliquots of seeds were put in Magenta vessels (Sigma Aldrich, St. Louis, USA) with 250 ml of deionized water. These seeds were placed at 2^{o}C and could be stored for up to 8-10 months. For aged *S. alterniflora* seeds, freshly harvested seeds were stored in zipper bags and dried at 2^{o}C.

Dried *S. pectinata* seeds were purchased from Western Native Seed (Coaldale, CO, USA), harvested in 2007 and mailed to the laboratory (Louisiana State Univ., Baton Rouge, LA., USA). After the seeds arrived at the laboratory, they were immediately stored dry in Mason jars at -20^{o}C. For imbibed *S. pectinata* seeds, ten grams of seeds

were put in Magenta vessels with 250 ml of deionized water and stored at 2°C.

S. spartinae were harvested in September and October, 2008-2009 from Port Fourchon, Louisiana. Spikes of *S. spartinae* were clipped, sealed in plastic zipper bags and transported to laboratory. *S. spartinae* seeds were stripped off the spikes by hand, air dried at room temperature (22-24°C) on the laboratory bench for 1 week and then stored dry in Mason jars at -20^{o}C.

Cold Stratification, Germination and Viability Tests

Imbibed *S. alterniflora* and *S. pectinata* were stored at 2^{o}C for cold stratification to break dormancy. Filled seeds of *S. alterniflora*, *S. pectinata and S. spartinae* were selected on a light table for experiments. Three replicates were used for each germination test, in which twenty filled seeds were put on three pieces of germination paper (Anchor Paper Co., St. Paul, Minnesota, USA) in a plastic Petri dish containing 8 ml of distilled water. The seeds were secured by placing a Kim-Wipe^{TM} disposable tissue over them. The plastic Petri dishes were incubated at 27^{o}C for 14 days for the germination test and another 14 days for a viability test. Emergence of the shoots and radicles was recorded at 7 and 14 days. The seeds that failed to germinate after 14 days were forwarded to the viability test. In the viability test, the upper third of coleoptile of the seed was cut off with a razor blade, a procedure that breaks dormancy, if present (Cohn and Gatz, 2002; Chappell, 2008).

Drying Method

The flash drying unit is the same as described in Chappell (2008). The flash dryer consists of a Nalgene jar (Thermo Fisher Scientific, Rochester, USA), a 12 V (0.16 A) computer fan (Radioshack, Fort Worth, USA) connected to 12 V (1000 mA) power

adapter (Radioshack, Fort Worth, USA), $CaSO_4$ desiccant (W.A. Hammond Drierite Company, Xenia, USA) and a ball jar rim (Muncie, USA) lined with mesh as a seed holder. The seeds, placed on the computer fan, are rapidly dried by air that is pulled up by the computer fan. Fresh desiccant (*ca.* 30 g) was used for each dry down experiment. If desiccant became moisture saturated (indicated as pink color) during an experiment, it was replaced by fresh Drierite (blue color).

Extraction of Total Soluble and Heat-stable Proteins

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., St. Louis, USA) unless stated. Fifty seeds (0.1 g/dry weight) of *S. alterniflora or S. pectinata* or 250 seeds of *S. spartinae* were freeze-clamped in liquid nitrogen, and then were ground by a pestle in a mortar that was pre-chilled in dry ice. Ground powders were immediately transferred into a pre-cooled glass homogenizer, and 3 ml (*S. alterniflora and S. pectinata*) of extraction buffer {50mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, pH 7.5} (stored at 4^{o}C) or 2 ml (*S. spartinae*) were added into the homogenizer. Protease inhibitor cocktail (#P9599, stored at -20^{o}C) (0, 10, 20 or 50 µl) (0%, 0.2%, 0.4%, 1% v/v) was added for optimization of the minimum amount of protease inhibitor to completely stop protease activity in protein extract. The mixture was fully homogenized at 4^{o}C by 30 pestle strokes, and then transferred into a 10 ml clean plastic tube (Corning, Corning Co., Lowell, MA, USA) that was pre-cooled on ice. The homogenizer was washed with 2 ml of extraction buffer (*S. alterniflora and S. pectinata*) or 1 ml (*S. spartinae*), ensuring that the protein extracts were completely transferred. The total homogenate was centrifuged at 14,000g at 4^{o}C for 20 minutes. After centrifugation, supernatant was carefully transferred into another clean plastic tube with a

Pasteur pipette (VWR International), and then centrifuged again at 14,000g at 4^{o}C for 20 minutes. The final supernatant was carefully pipetted to a clean plastic tube and divided into two portions. One was used to measure the concentration of total soluble protein by the Bradford method (Bradford, 1976). The other was used to verify the optimum heating temperature and duration. One milliliter of the supernatant was incubated at 75^{o}C, 85^{o}C or 95^{o}C for 10, 20, 30, 40, 60 or 90 minutes for heating temperature and duration optimization. After heating, the tubes were buried in ice for 0, 10, 20 and 30 minutes, and then centrifuged at 14,000g, 16,000g, 18,000g and 20,000g, at 4^{o}C for 10, 30, 40 and 60 minutes for optimization of cold incubation of heated protein extracts and centrifugation speed and duration. Each supernatant was carefully pipetted out with Pasteur pipettes into clean plastic tubes, and protein concentration was measured by Bradford procedure (Bradford, 1976).

A standard Bradford assay (B6916), with 0.05 ml of the protein sample and 1.5 ml of the Bradford reagent per tube, was used for the protein concentration determination. A standard curve was created when every new bottle of the reagent was used. For the standard curve, seven standard solutions containing 0, 0.1, 0.25, 0.5, 0.75, 1 and 1.4 mg/ml bovine serum albumin (BSA) were prepared. BSA was dissolved in the same HEPES buffer as for protein extraction. HEPES buffer without any protein was used as a blank. Bradford reagent was stored at 4^{o}C, gently inverted several times to mix, and warmed up to room temperature before experimental measurements. The glass tube was gently vortexed after addition of the protein sample and Bradford reagent. The mixture of protein sample and Bradford reagent was transferred to a 1.5 ml disposable plastic cuvette. The absorbance was recorded at 595 nm using a spectrophotometer (UV-2450,

Shimazu Scientific Instruments, Columbia, MD, USA) after a 5-minute colorimetric reaction (Fig. A-3). The seed protein samples were properly diluted, ensuring that they were within the linear range of 0.1-1.4 mg/ml of the standard curve. The recorded absorbance was entered into SigmaPlot (Version 11.0) to graph the standard curve (Figs. A-1 to A-2), from which a linear regression equation was obtained. The protein concentration of seed samples was determined by calculating the absorbance from the linear regression equation. A sample calculation and standard curves are presented in Appendix A.

PDQ^{TM} Protease Assay

The reagent vials of the PDQ^{TM} assay (AthenaES, Environmental Science Inc., Baltimore, USA), consisting of an upper storage solution and bottom matrix substrate (stored at 2^{o}C), were warmed to room temperature before experiments. After the upper storage solution was poured off, 0.5 ml of each control solution or test sample solution was added to the vial and incubated for 3 hours at 37^{o}C. After incubation, 1.5 ml of 0.2 N NaOH was added to each vial to amplify the color, and then transferred to standard cuvettes. One and half milliliters of 0.2 N NaOH added to 0.5 ml of Tris-HCl buffer (pH 8.0) was used as the blank control. Absorbance was recorded at 450 nm. A standard curve for the PDQ colorimetric assay (Fig. A-5) was constructed by measuring the absorbance at 450 nm of a ten-fold dilution series of trypsin (Tris-HCl buffer, pH 8.0) {0.4 BAEE [(N-benzoyl-L-arginine ethyl ester) units (one BAEE unit of trypsin is the amount of enzyme causing an increase in absorbance of 0.001 per minute at 25^{o}C and 253 nm)/ml]-400 BAEE units/ml}. The procedure described by the manufacturer was used for the assay (http://www.athenaes.com/datasheet_pdq.php).

Electrophoresis

TCA (trichloroacetic acid) (100%) was added to solutions of either the total or heat-stable fraction to make a final concentration of 10% TCA (w/v). The mixture was buried in ice overnight (*ca.* 12 hours). The cold-incubated mixture was centrifuged at 16,000g at 4^{o}C for 30 minutes. The supernatant was carefully decanted after centrifugation. The pellet was washed with cold acetone (-20^{o}C) and centrifuged at 16,000g at 4^{0}C for 10 minutes. The supernatant was carefully pipetted and discarded. The washing process was repeated three times. The pellet was vacuum dried (Speed Vac, SAVANT) and then dissolved in 100 µl of rehydration solution [7M urea (Bio-Rad Laboratories, USA), 2M thiourea (Bio-Rad Laboratories, USA), 4% CHAPS (w/v) (Bio-Rad Laboratories, USA) and 20mM dithiothreitol (DTT) (GE Healthcare Life Science, USA)] at room temperature overnight. Fifty microliters of the rehydration buffer was pipetted out and measured for protein concentration using rehydration buffer as the blank (Bradford, 1976). For SDS-PAGE, 10 ml of resolving gel solution [2.5 ml of 1M Tris-HCl (pH8.8), 100 µl of 10% SDS (w/v) (Bio-Rad Laboratories, USA), 3 ml of 40% acrylamide (w/v) (Bio-Rad Laboratories, USA), 50 µl of 10% (w/v) ammonium persulfate (APS) and 5µl tetramethylethylenediamine (TEMED)] and 10 ml of stacking gel solution [2.5 ml of 1M Tris-HCl (pH 6.8), 100 µl of 10% SDS (w/v), 1.3 ml of 40% acrylamide, 50 µl of 10% APS (w/v) and 10µl tetramethylethylenediamine (TEMED)] (APS and TEMED were not added until gels were ready to pour) were prepared. The protein sample solution was mixed with 2x SDS-PAGE sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol (v/v), 4% SDS (w/v) and 0.1% bromphenol blue (w/v)] at a ratio of 1:1, and heated for 5 min at 95^{o}C in a water bath. After the 12% gel was done, 20 µg of

MW-marker (Sigma M3913) or protein sample solution was carefully pipetted into gel sample well. Proteins were first concentrated in the stacking gel at a constant voltage of 60V for around 30 minutes at room temperature, and then separated in the resolving gel at a constant voltage of 110V for 90 minutes at room temperature. Gels were stained with 0.1% Coomassie Blue R-250 (10% acetic acid and 40% methanol) for 6 hours on an agitator and were de-stained with a solution of 15% (v/v) methanol and 10% (v/v) acetic acid for up to 10 hours. After de-staining, gels were washed three times with distilled water, and then the gel profiles were visualized and scanned with Magicscan (version 4.2) (UMAX, Techville, Inc., TX, USA).

Statistical Analysis

Standard error was calculated and expressed for each mean. For pairwise comparisons within the same experiment, statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak in SigmaPlot (version 11.0) (significance level is $\alpha=0.05$). For comparison of two independent experiments, statistical significance was determined by Student's *t*-test in SigmaPlot (version 11.0) (significance level is $\alpha=0.05$).

RESULTS

Optimum Heating Temperature and Duration

Since the experiments determining the optimum heating duration were conducted before the other optimization experiments, the centrifugation speed and duration used for this section was 14,000 g for 40 min instead of the optimum speed of 20,000 g for 40 min. Thus, protein concentration of heat-stable fractions in Figs. 2.2-2.11 is only comparable to protein concentrations of 14,000 g fractions in Figs. 2.14-2.23.

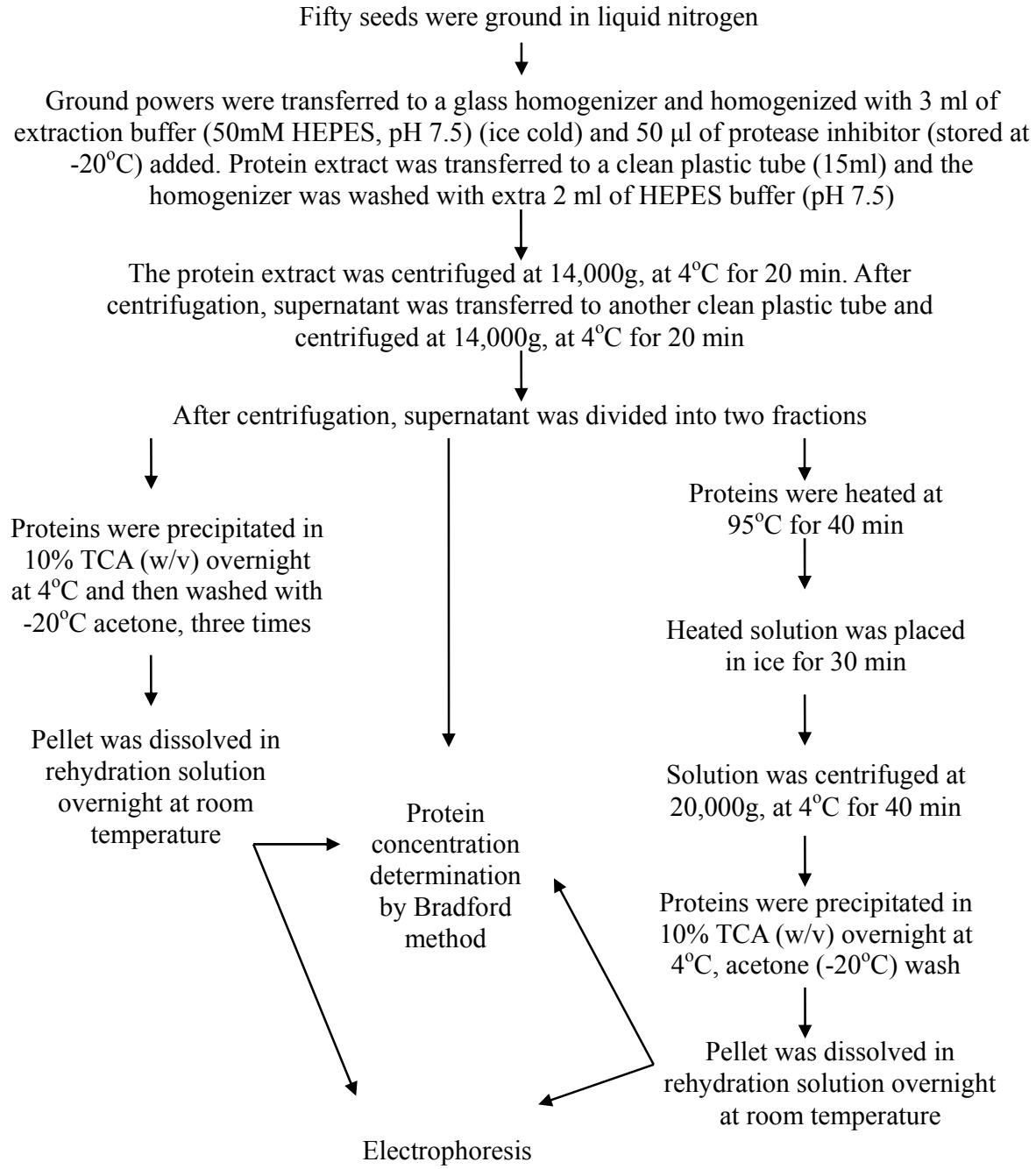


Figure 2.1. Flow chart of protein extraction procedures

Table 2.4 gives a summary of contents from Figures 2.2-2.11. The protein concentration was expressed as µg/seed or µg/embryo instead of µg/µl because µg/seed or µg/embryo is the standard expression used in most seed biology reports, allowing comparisons of the protein concentration of seed/embryo across different studies. Figures 2.2-2.11 confirm that some soluble proteins were heat-denatured. The trend of a rapid protein precipitation starting during the first 10 min was observed consistently in Fig. 2.2-2.11. At the beginning of protein precipitation, the concentration of soluble protein decreased more rapidly at 95^{o}C than at 75^{o}C and 85^{o}C (Figs. 2.2-2.11). The speed of protein precipitation gradually slowed down after 10 minutes, and reached a constant level by 40 min at 75^{o}C, 85^{o}C and 95^{o}C (Figs. 2.2-2.11). A continued heating after 40 min did not significantly denature more soluble protein at these three temperatures. Thus, heating at 95^{o}C for at least 40 minutes is needed to obtain a heat-stable protein fraction from the *S. alterniflora*, *S. pectinata* and *S. spartinae* seeds.

Optimum Duration for Cold Precipitation

Table 2.5 gives a summary of key values from Figures 2.12-2.13. Both Figure 2.12 and Figure 2.13 show that soluble protein concentrations of heat-stable fractions stabilized after ice incubation for 20 minutes. Cold precipitation for 30 minutes was sufficient to precipitate the heat-unstable protein in both the *S. pectinata* and *S. alterniflora* seeds.

Optimum Centrifugation Speed and Duration

Table 2.6 shows the summary of contents of Figures 2.14-2.23. The amount of heat-soluble proteins decreased as the centrifugation duration was extended, as shown in Figs 2.14-2.23. The rapid decrease of heat-stable proteins indicated the facilitation of

Table 2.4. A summary of key values from Figures 2.2-2.11. SA: *Spartina alterniflora*; SP: *Spartina pectinata*; SS: *Spartina spartinae*; FH: fully hydrated; FD: flash-dried; ND: non-dormant; D: dormant; HS: heat-stable; MC: moisture content (DWB); [Total Fraction]: protein concentration of total seed extract before heating; [HS Fraction]: protein concentration of heat-stable fraction after heating at 95^{o}C for 40 minute. *: one biological replicate and three technical replicates for *Spartina spartinae* seeds. Protein values ± SE (standard error).

Figure #	**Species**	**[Total Fraction] (µg/seed or µg/embryo)**	**[HS Fraction] (µg/seed or µg/embryo)**	**Seed Tissue**	**Harvest Year**	**Germination (%)**	**Viability (%)**	**MC (%)**
Fig. 2.2	FH & ND SA	49.2 ± 0.9	28.7 ± 1.5	Whole seed	2007	95	95	121
Fig. 2.3	FH & D SA	53.8 ± 1	39.8 ± 0.7	Whole seed	2007	0	90	149
Fig. 2.4	FD & ND SA	36.8 ± 1.1	14.8 ± 0.3	Whole seed	2007	0	0	15
Fig. 2.5	FD & D SA	37.8 ± 0.4	17.1 ± 0.6	Whole seed	2008	0	5	18
Fig. 2.6	Dry & Aged SA	34.0 ± 2	9.1 ± 0.6	Whole seed	2007	0	0	9
Fig. 2.7	FH & ND SA	36.0 ± 0.8	9.1 ± 0.9	Embryo	2008	95	95	140
Fig. 2.7	FH & D SA	33.6 ± 1.2	8.6 ± 0.3	Embryo	2008	0	95	124
Fig. 2.8	FH & ND SP	51.2 ± 0.7	30.4 ± 1.0	Whole seed	2007	90	90	127
Fig. 2.9	FH & D SP	56.3 ± 0.5	37.7 ± 1.5	Whole seed	2007	0	95	131
Fig. 2.10	Dry & D SP	48.8 ± 1.5	14.5 ± 0.9	Whole seed	2007	0	88	10
Fig. 2.11	Dry & D SS*	50.6 ± 0.9	13.5 ± 0.5	Whole seed	2008	0	60	6

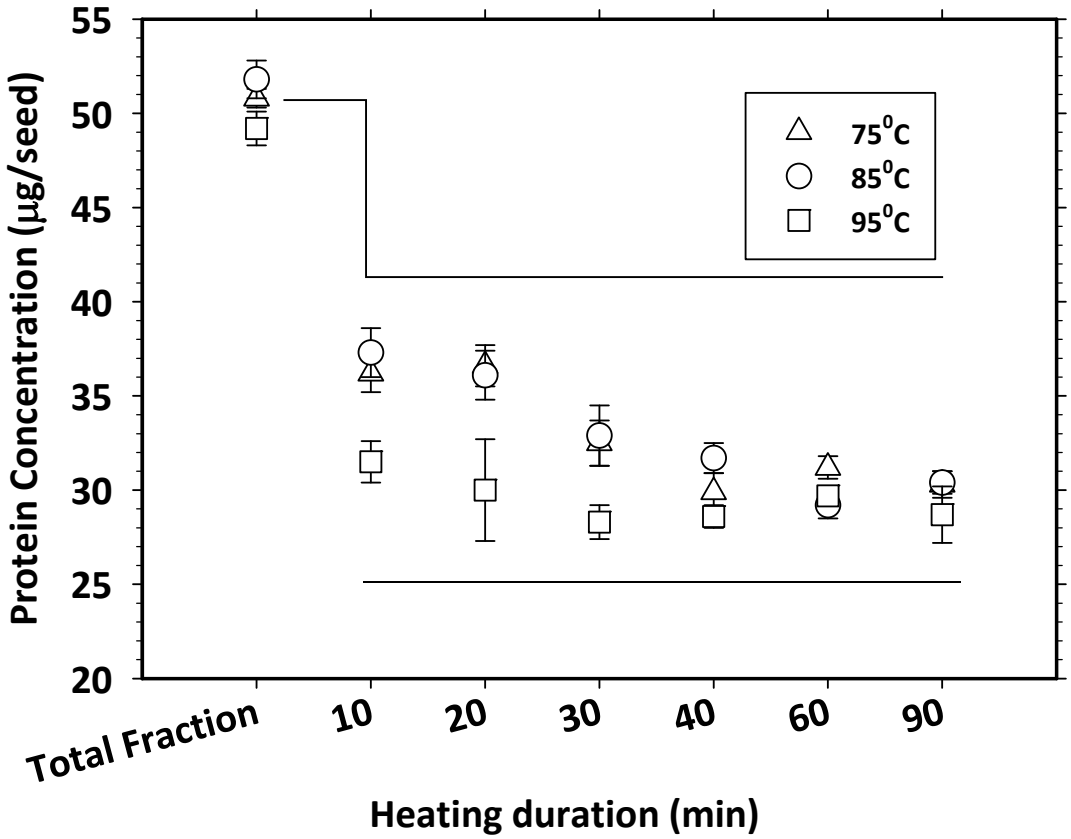


Figure 2.2. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for fully-hydrated and non-dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2°C [Moisture content (MC) =121% (DWB), Germination (G) =95%, Viability (V) =95%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

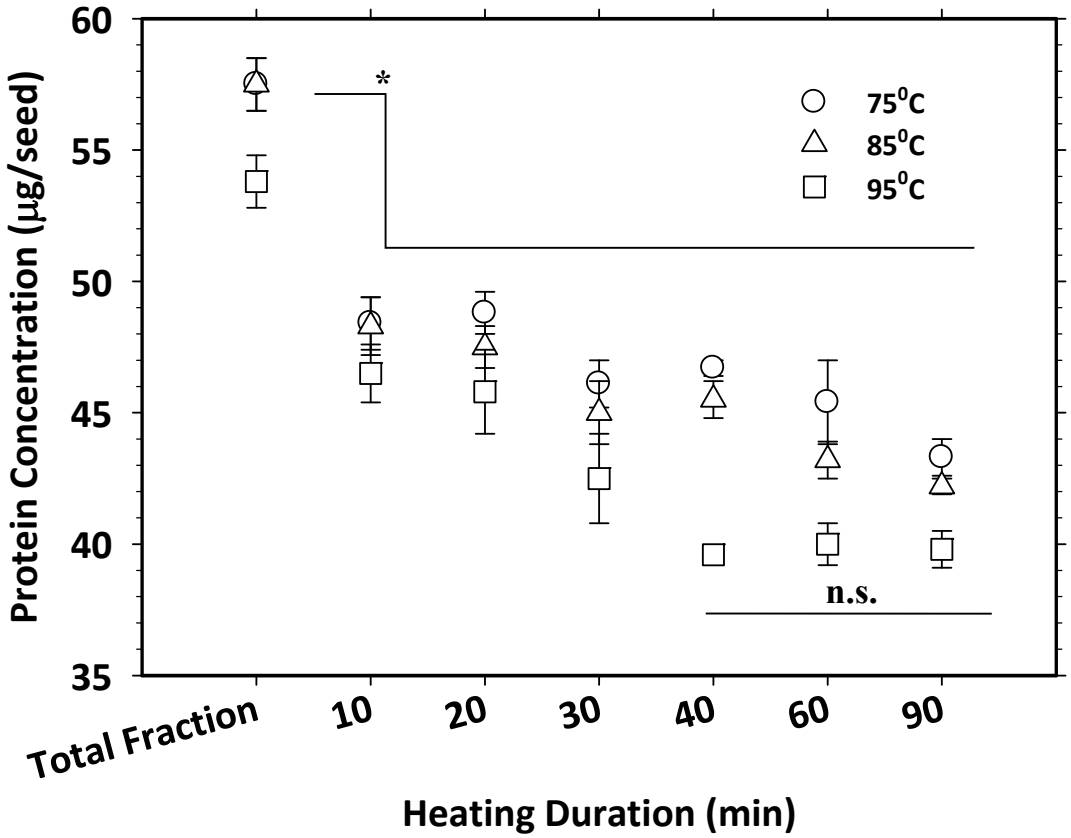


Figure 2.3. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for fully-hydrated and dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007), cold stratified for 2 weeks at 2^{o}C [MC=149% (DWB), G=0%, V=90%], flash frozen in liquid N_2 and stored at -80^{0}C for 6 months. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

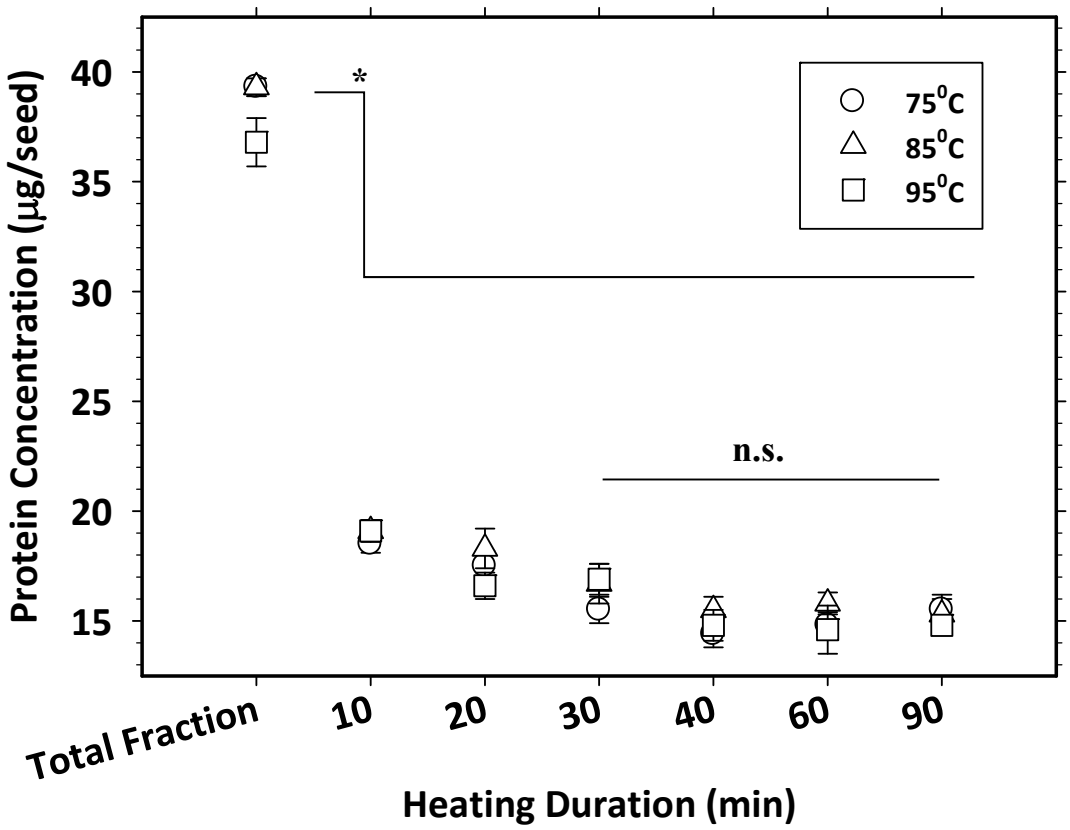


Figure 2.4. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for 7-day-flash dried and non-dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2°C [MC=15% (DWB), G=0%, V=0%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

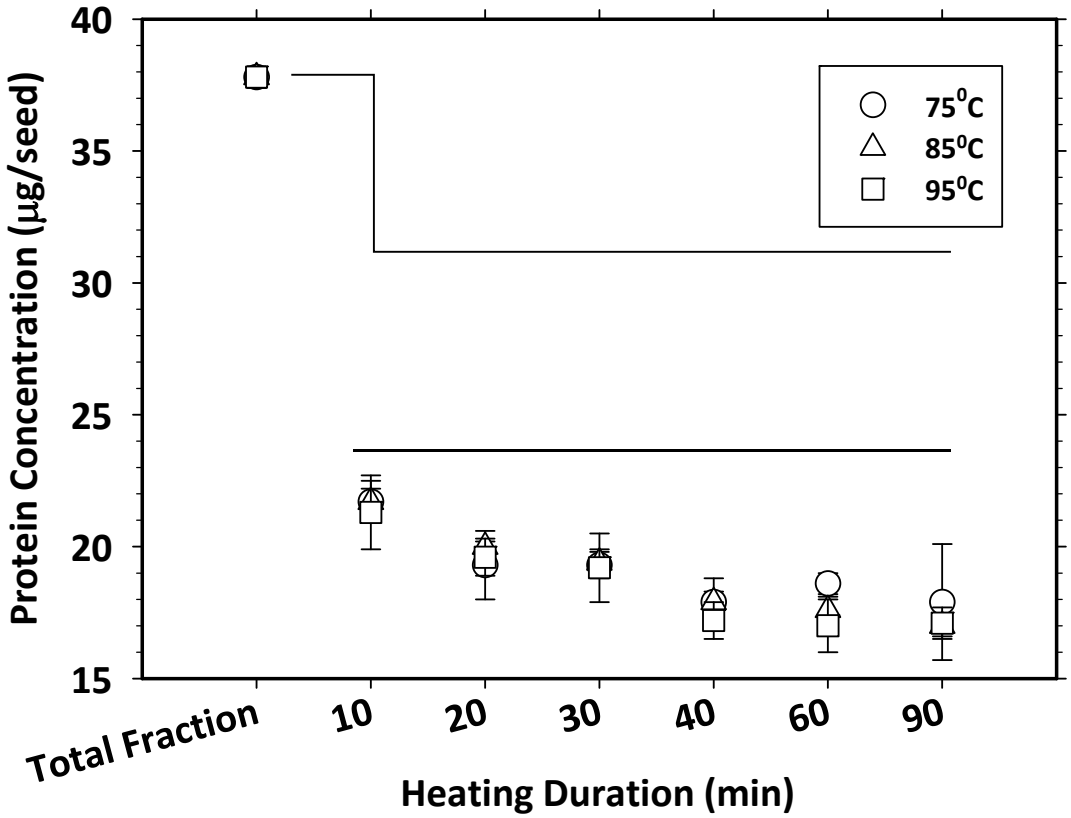


Figure 2.5. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for 7-day-flash dried and dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008), cold stratified for 1.5 months at 2°C [MC=18% (DWB), G=0%, V=5%], flash frozen in liquid N_2 and stored at -80°C for 6 months. Each mean represents 1 biological replicate and 3 analytical replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

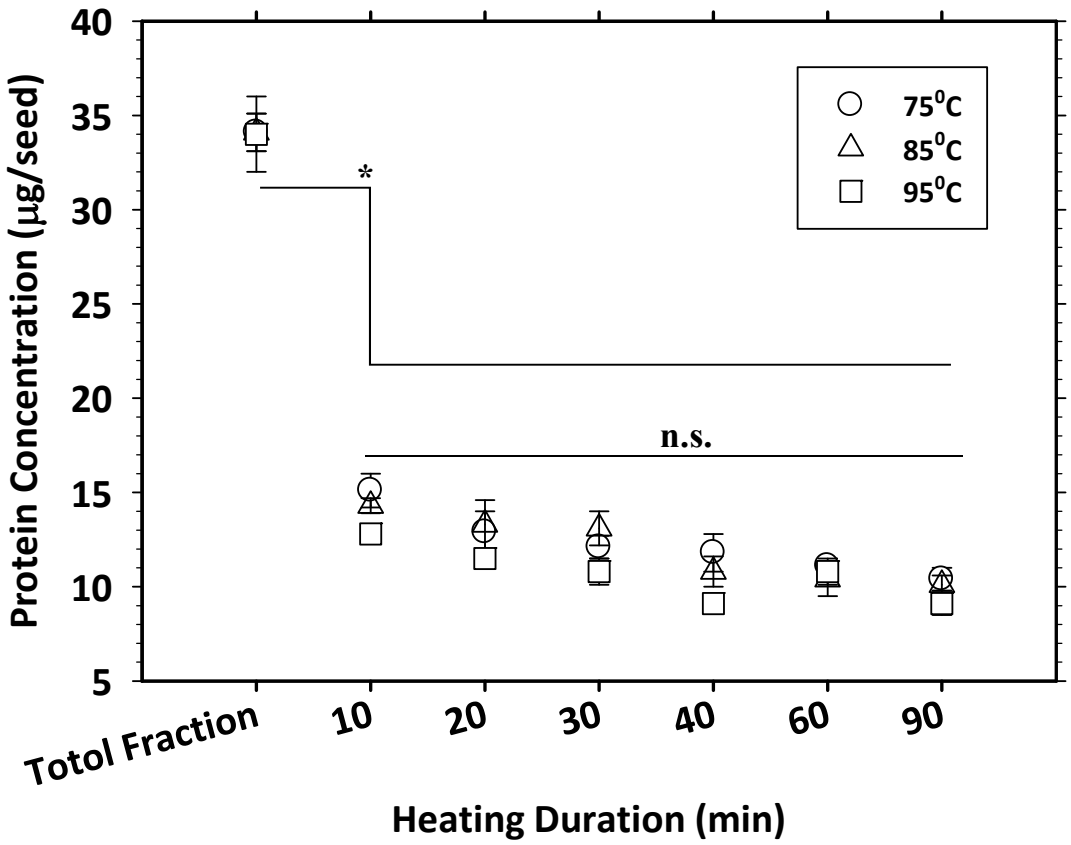


Figure 2.6. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for dry and aged *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and stored dry for 6 months at 23°C [MC=9% (DWB), G=0%, V=0%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

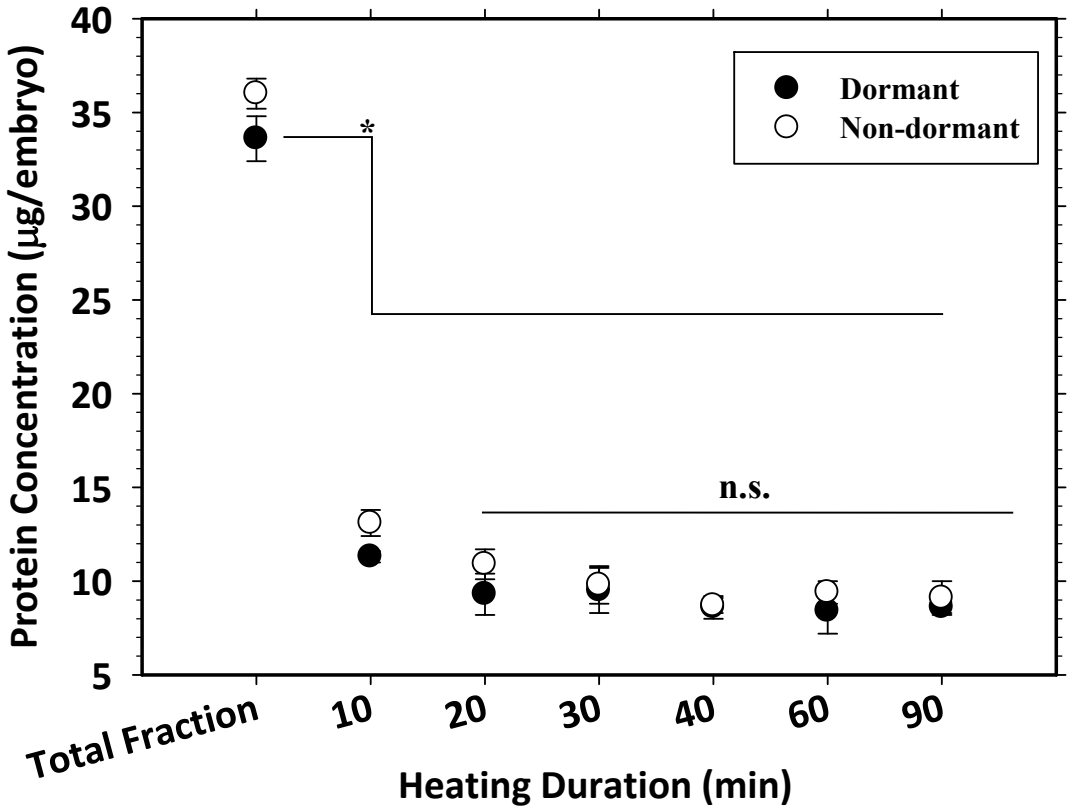


Figure 2.7. Protein concentrations of total and heat-stable fractions at 95°C for different durations for fully-hydrated and dormant/non-dormant *S. alterniflora* isolated embryos. Dormant: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008), cold stratified for 1 month at 2°C [MC (whole seed) =124% (DWB), G=0%, V=95%], flash frozen in liquid N_2 and cut to isolate the embryos. Non-dormant: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008), cold stratified for 9 months at 2^0C [MC (whole seed) =140% (DWB), G =95%, V =95%], flash frozen in liquid N_2 and cut to isolate the embryos. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

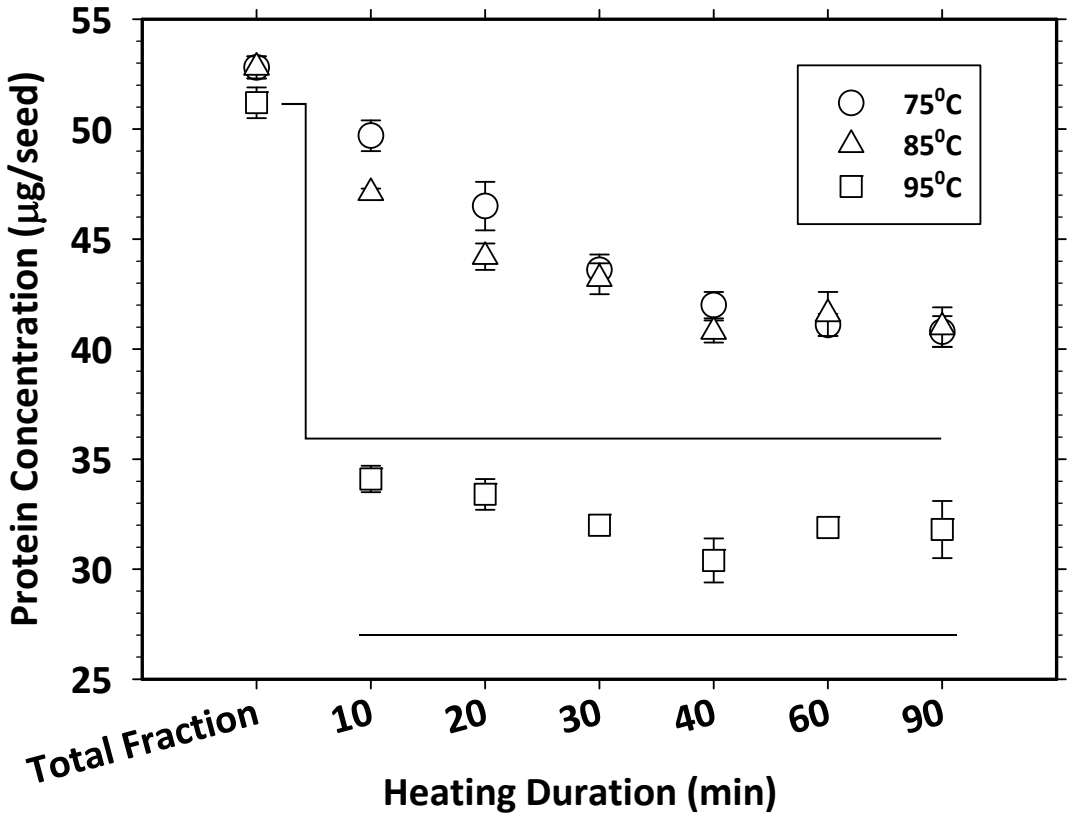


Figure 2.8. Protein concentrations of total fractions and heat-stable fractions that were at different heating temperatures for different durations for fully-hydrated and non-dormant *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), stored dry at -20°C for 5 months, and cold stratified at 2°C for 3 months [M =127% (DWB), G=90%, V=90%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

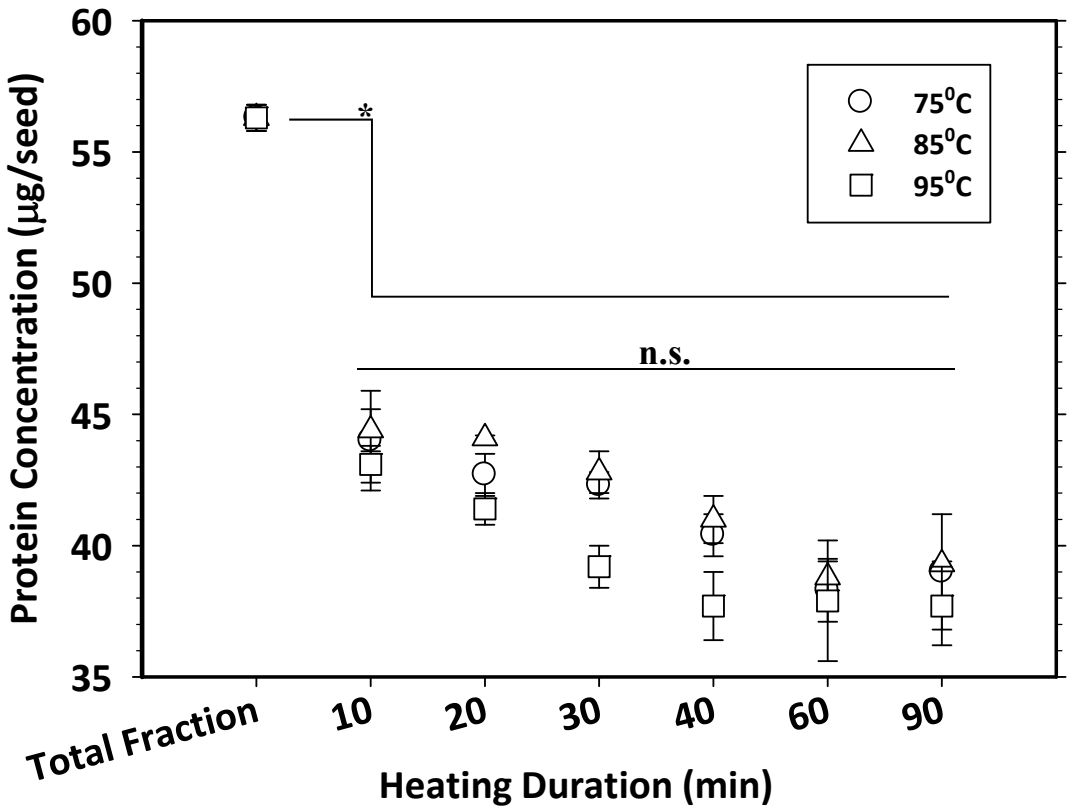


Figure 2.9. Protein concentrations of total fractions and heat-stable fractions that were at different heating temperatures for different durations for fully-hydrated and dormant *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), stored dry at -20°C for 7 months, and cold stratified at 2°C for 1 week [MC=131% (DWB), G=0%, V=95%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

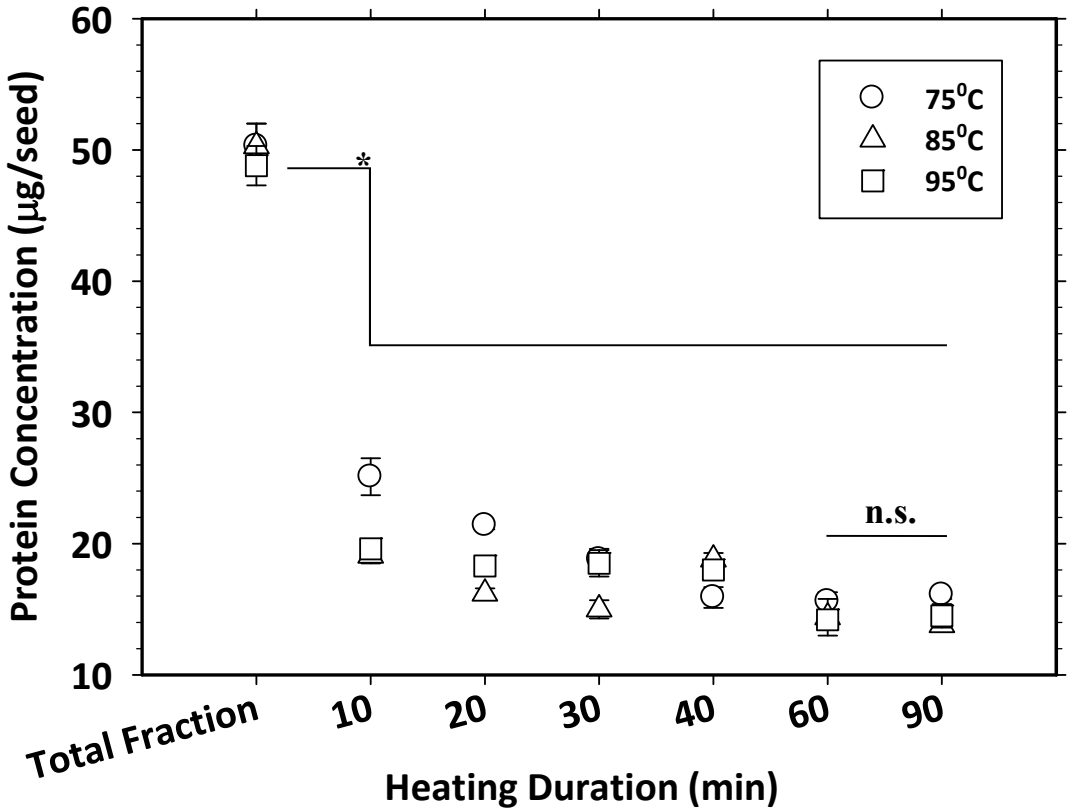


Figure 2.10. Protein concentrations of total fractions and heat-stable fractions that were at different heating temperatures for different durations for dry and dormant *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), stored dry at -20°C for 7 months [MC=10% (DWB), G=0%, V=88%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

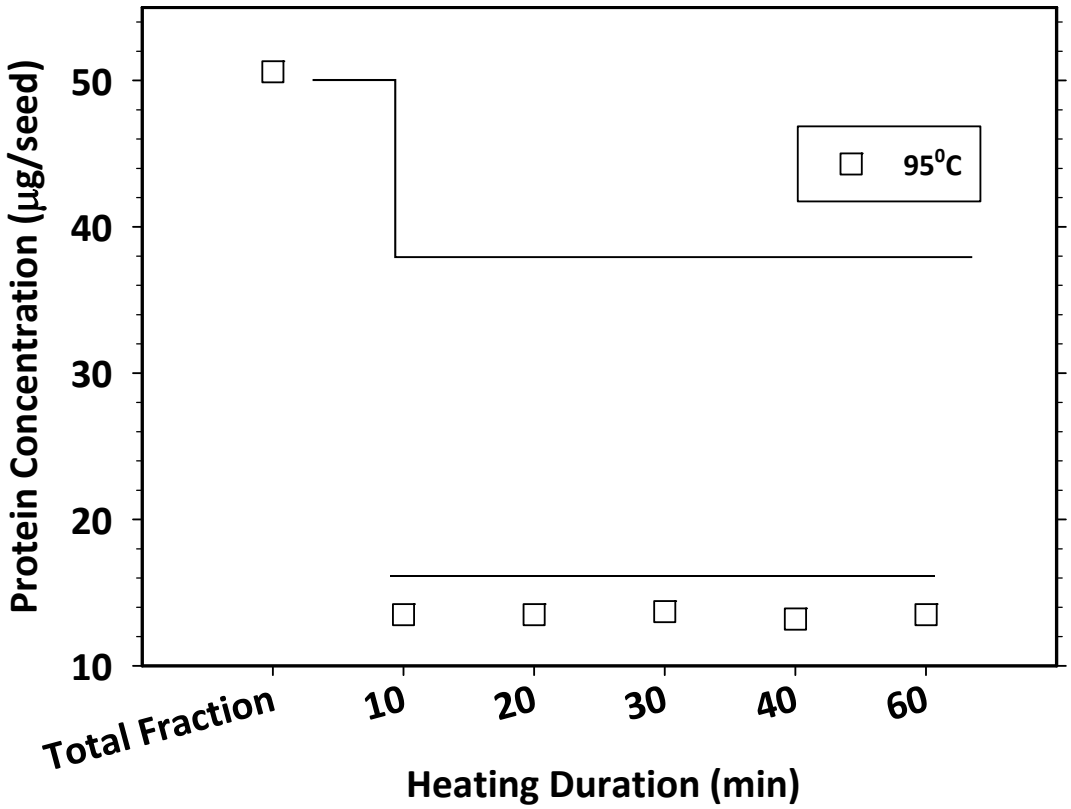


Figure 2.11. Protein concentrations of total fractions and heat-stable fractions that were at 95°C for different durations for dry and dormant *S. spartinae* seeds. *S. spartinae* seeds were harvested from Port Fourchon, LA (2008), flash frozen in liquid nitrogen [MC=6% (DWB), G=0%, V=60%] and stored at -80°C. Each value represents 1 biological replicate. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

denatured protein precipitation by centrifugation. For any certain time, denatured proteins were precipitated more rapidly at 20,000 g and least at 14, 000g. The concentration of heat-soluble proteins reached a constant value by 60 min at 14,000 g and16, 000 g, but by 40 min at 20,000g. Thus, a centrifugation speed of 20,000 g for 40 minutes is needed to obtain the heat-stable fraction in both the *S. pectinata* and *S. alterniflora* seed/embryo.

Percentage of Heat-stable Protein Fractions and Desiccation Tolerance

Percentages of heat-stable protein fractions of *S. alterniflora*, *S. pectinata* and *S. spartinae* under differential physiological states are shown in Figure 2.24. Desiccation tolerant *S. pectinata* has a significantly higher heat-stable fraction than desiccation sensitive *S. alterniflora* in fully hydrated, dormant (68% ± 2 vs. 54% ± 3, $p \leq 0.05$) and non-dormant (59% ± 4 vs. 31% ± 2, $p \leq 0.01$) seeds; however, desiccation tolerant dry and dormant *S. pectinata* has a much smaller heat-stable fraction than desiccation intolerant *S. alterniflora* of air dried, fully hydrated, dormant and non-dormant seeds (20% ± 1 vs. 31% ± 3 & 54% ± 3 & 31% ± 2, $p \leq 0.05$).

The percentage of heat-stable fractions is affected by the physiological states of seeds. Heat-stable fractions of dormant seeds were significantly higher than non-dormant seeds in both fully hydrated *S. alterniflora* and *S. pectinata* (*S. alterniflora*, 54% ± 3 vs. 31% ± 2; *S. pectinata*, 68% ± 2 vs. 59% ± 4; $p \leq 0.05$). Heat-stable fractions of fully hydrated *S. alterniflora* and *S. pectinata* seeds were significantly higher than dry seeds (*S. alterniflora*, 54% ± 3 vs. 31% ± 4; *S. pectinata*, 68% ± 2 vs. 20% ± 1; $p \leq 0.01$). The whole seeds of *S. alterniflora* have a higher heat-stable fraction than embryos (dormant, 54% ± 3 vs. 29% ± 2; non-dormant, 43% ± 3 vs. 30% ± 3, $p \leq 0.01$).

Table 2.5. A summary of key values from Figures 2.12-2.13 (ice precipitation time). SA: *Spartina alterniflora*; SP: *Spartina pectinata*; FH: fully hydrated; ND: non-dormant; D: dormant; HS: heat-stable; MC: moisture content (DWB); [Total Fraction]: protein concentration of total seed extract before heating; [HS Fraction]: protein concentration of heat-stable fraction after heating at 95^{o}C for 40 minutes.

Figure #	Species	[Total Fraction] (µg/seed or µg/embryo)	[HS Fraction] (µg/seed or µg/embryo)	Seed Tissue	Harvest Year	Germination (%)	Viability (%)	MC (%)
Fig. 2.12	FH & ND SA	52.9 ± 0.7	31.2 ± 0.8	Whole seed	2007	95	95	121
Fig. 2.12	FH & D SA	57.5 ± 1	42.8 ± 0.6	Whole seed	2007	0	90	149
Fig. 2.12	Dry & Aged SA	35.9 ± 0.7	11.4 ± 1.2	Whole seed	2007	0	0	9
Fig. 2.12	FH & ND SA	34.6 ± 0.4	22.7 ± 0.1	Embryo	2007	90	95	121
Fig. 2.12	FH & D SA	33.1 ± 0.7	22.5 ± 0.1	Embryo	2007	0	90	149
Fig. 2.13	FH & ND SP	51.3 ± 0.4	13.7 ± 0.7	Whole seed	2007	85	85	123
Fig. 2.13	FH & D SP	52.1 ± 0.5	12.8 ± 0.4	Whole seed	2007	0	85	132
Fig. 2.13	Dry & D SP	50.3 ± 1.7	12.9 ± 0.7	Whole seed	2007	0	88	10

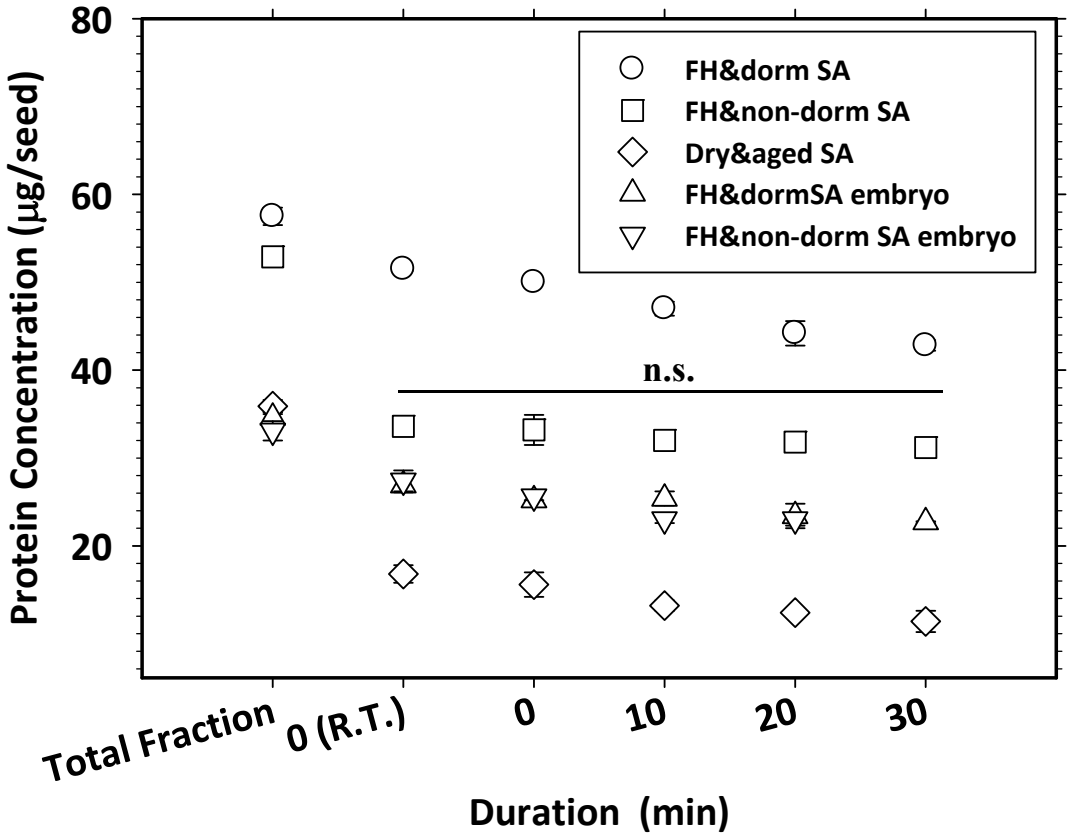


Figure 2.12. Protein concentrations of total fractions and heat-stable proteins prepared with different ice precipitation times for *S. alterniflora* seeds/embryos. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007). Fully-hydrated and dormant: Cold stratified for 2 weeks at 2°C [MC=149% (DWB), G=0%, V=90%]. Fully-hydrated and non-dormant : Cold stratified for 6 months at 2°C [MC=121% (DWB), G=95%, V=95%]. Dry and aged: Stored dry for 6 months at 23°C [MC=9% (DWB), G=0%, V=0%]. Fully-hydrated and dormant embryo : Cold stratified for 2 weeks at 2°C [MC (whole seed) = 149% (DWB), G= 0%, V=90%], flash frozen in liquid N_2 and cut to isolate the embryos. Fully-hydrated and non-dormant embryo: Cold stratified for 7 months at 2°C [MC=121% (whole seed), G=90%, V=95%], flash frozen in liquid N_2 and cut to isolate the embryos. Total fraction: protein extracts that were without heat treatment; 0 (R.T.): protein extracts without cold precipitation were centrifuged at room temperature after heated at 95°C for 40 min; 0: protein extracts without cold precipitation were centrifuged at 4°C after heating; 10, 20, 30: proteins with cold precipitation were centrifuged at 4°C after heating. For whole seeds, each mean represents 3 biological replicates. For embryos, each mean represents 1 biological replicate and 3 analytical replicates. Error bars ± SE. n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

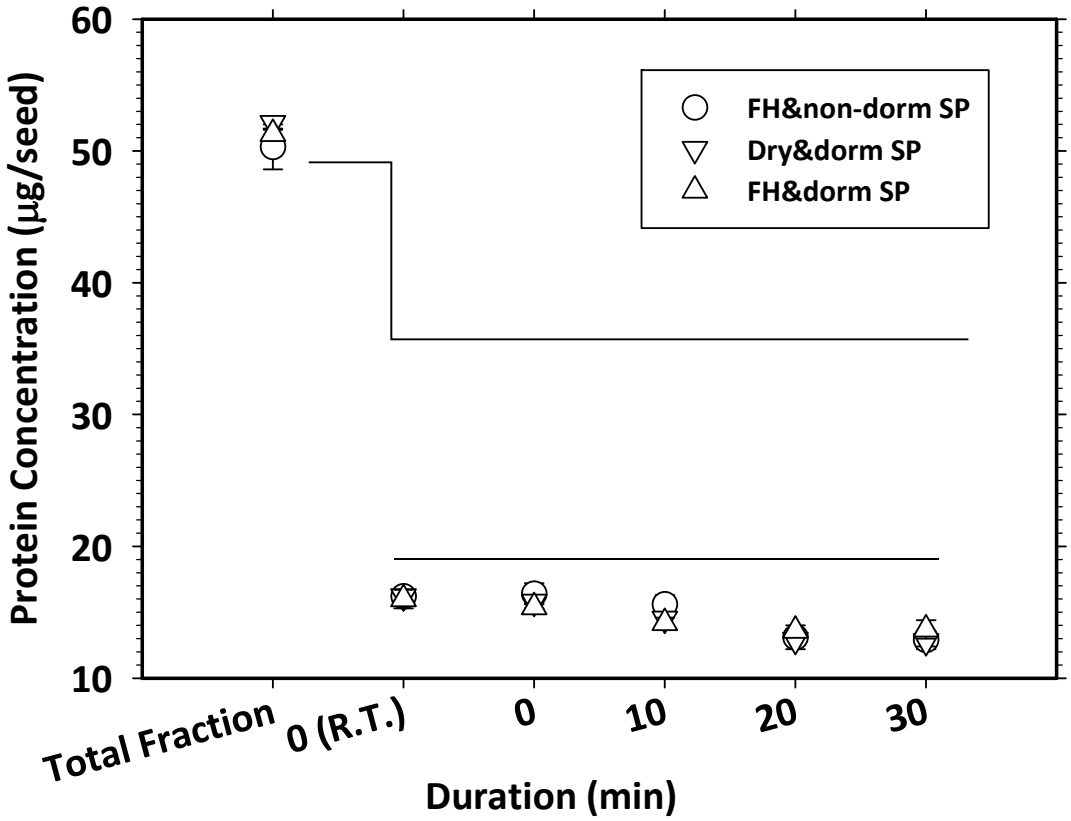


Figure 2.13. Protein concentrations of total fractions and heat-stable proteins prepared under different ice precipitation times for *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007). Fully-hydrated and non-dormant: stored dry at -20^{0}C for 17 months, and cold stratified at 2^{o}C for 3 months [MC=123% (DWB), G=85%, V=85%]. Dry and dormant: stored dry at -20^{0}C for 7 months. [MC=10% (DWB), G=0%, V=88%]. Fully-hydrated and dormant: stored dry at -20^{0}C for 20 months, and cold stratified at 2^{0}C for 1 week [MC=132% (DWB), G=0%, V=85%]. Total fraction: protein extracts that were without heat treatment; 0 (R.T.): protein extracts without cold precipitation were centrifuged at room temperature after heating; 0: protein extracts without cold precipitation were centrifuged at 4^{o}C after heating; 10, 20, 30: proteins with cold precipitation were centrifuged at 4^{o}C after heating. Each mean represents 3 biological replicate. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

Table 2.6. A summary of key values from Figures 2.14-2.23 (Centrifugation speed optimization). SA: *Spartina alterniflora*; SP: *Spartina pectinata*; FH: fully hydrated; FD: flash-dried; ND: non-dormant; D: dormant; HS: heat-stable; MC: moisture content (DWB); [Total Fraction]: protein concentration of total seed extract before heating; [HS Fraction]: protein concentration of heat-stable fraction after heating at 95°C for 40 minutes.

Figure #	Species	[Total Fraction] (µg/seed or µg/embryo)	[HS Fraction] (µg/seed or µg/embryo)	Seed Tissue	Harvest Year	Germination (%)	Viability (%)	MC (%)
Fig. 2.14	FH & ND SA	54.9 ± 1.5	17.1 ± 0.7	Whole seed	2007	95	95	133
Fig. 2.15	FH & D SA	61.5 ± 0.4	31.8 ± 0.7	Whole seed	2007	0	90	149
Fig. 2.16	FD & ND SA	38.4 ± 1.2	18.4 ± 1.0	Whole seed	2007	0	5	14
Fig. 2.17	FD & D SA	32.7 ± 1.1	18.9 ± 0.6	Whole seed	2007	0	5	18
Fig. 2.18	Dry & Aged SA	40.3 ± 2.6	10.2 ± 0.3	Whole seed	2007	0	0	9
Fig. 2.19	FH & ND SA	37.9 ± 1.0	12.4 ± 0.1	Embryo	2007	90	90	139
Fig. 2.20	FH & D SA	29.3 ± 0.4	13.7 ± 0.6	Embryo	2007	0	95	121
Fig. 2.21	FH & ND SP	39.7 ± 0.9	24.8 ± 0.4	Whole seed	2007	80	85	114
Fig. 2.22	FH & D SP	52.8 ± 0.5	35.1 ± 1.7	Whole seed	2007	0	85	143
Fig. 2.23	Dry & D SP	49.1 ± 1.6	9.7 ± 0.8	Whole seed	2007	0	88	10

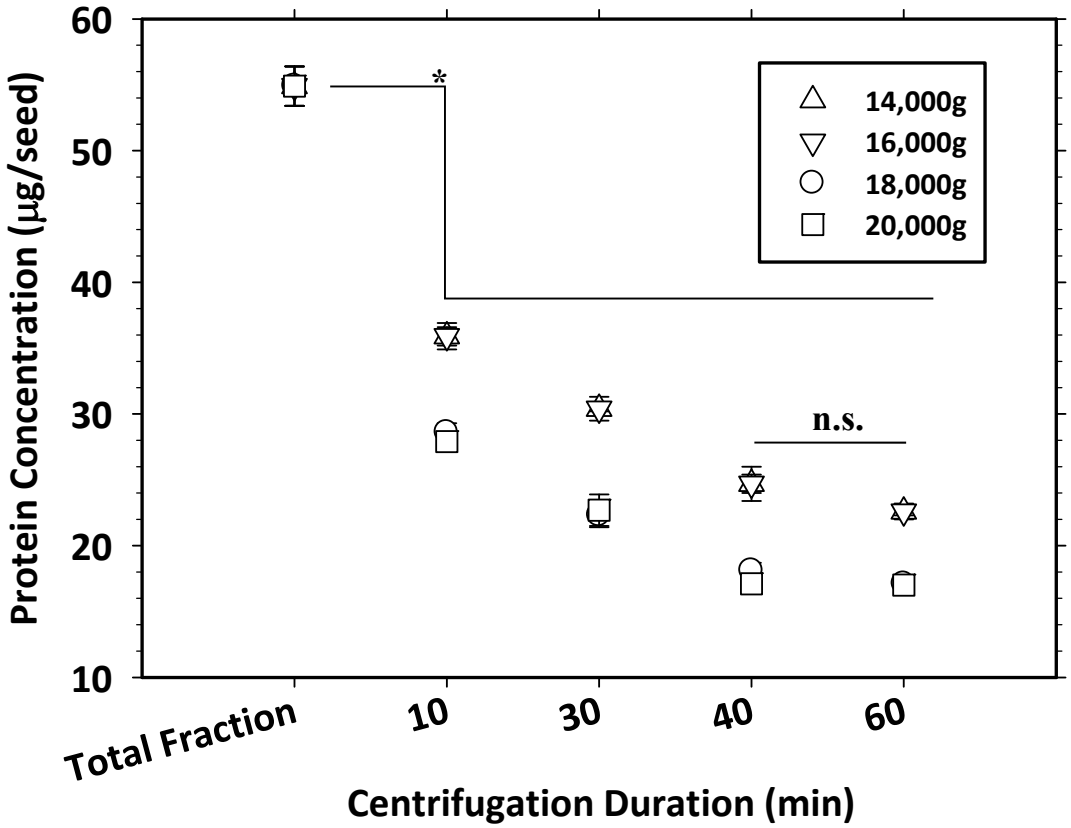


Figure 2.14. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for fully-hydrated and non-dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2^{o}C [MC=133% (DWB), G=95%, V=95%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

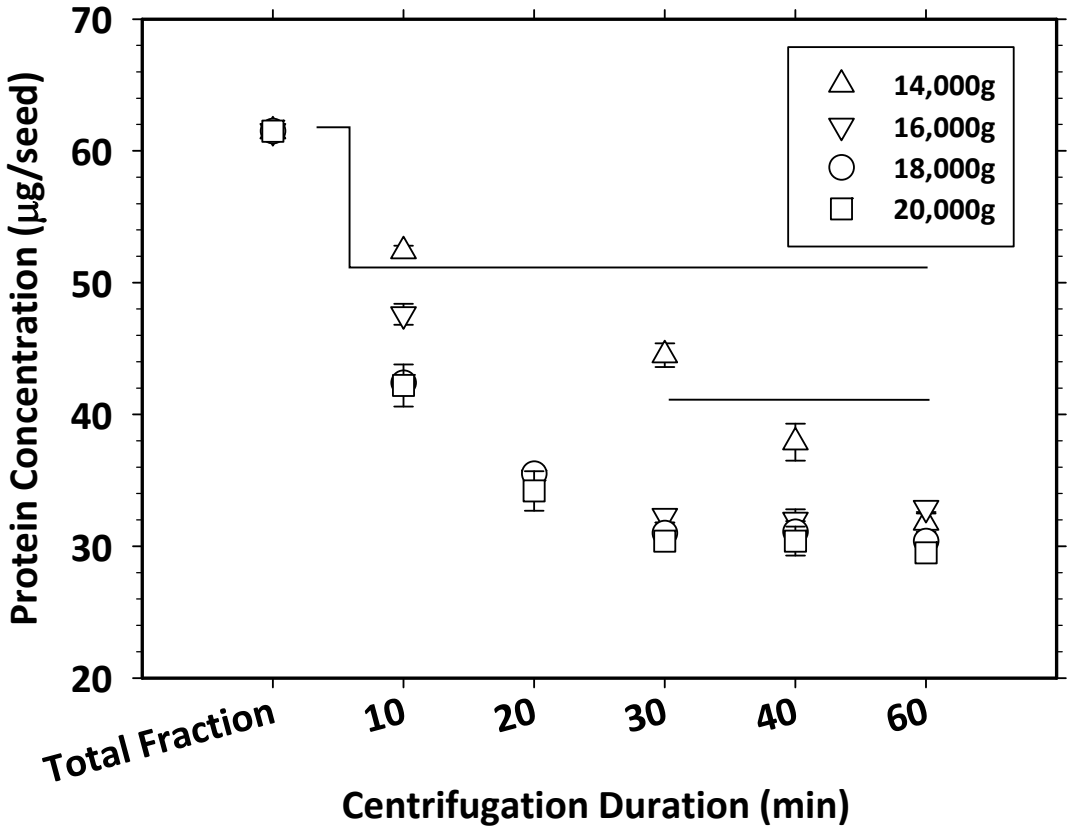


Figure 2.15. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for fully-hydrated and dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007), cold stratified for 2 weeks at 2^{o}C [MC=149% (DWB), G=0%, V=90%], flash frozen in liquid N_2 and stored at -80^{0}C for 6 months. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05)

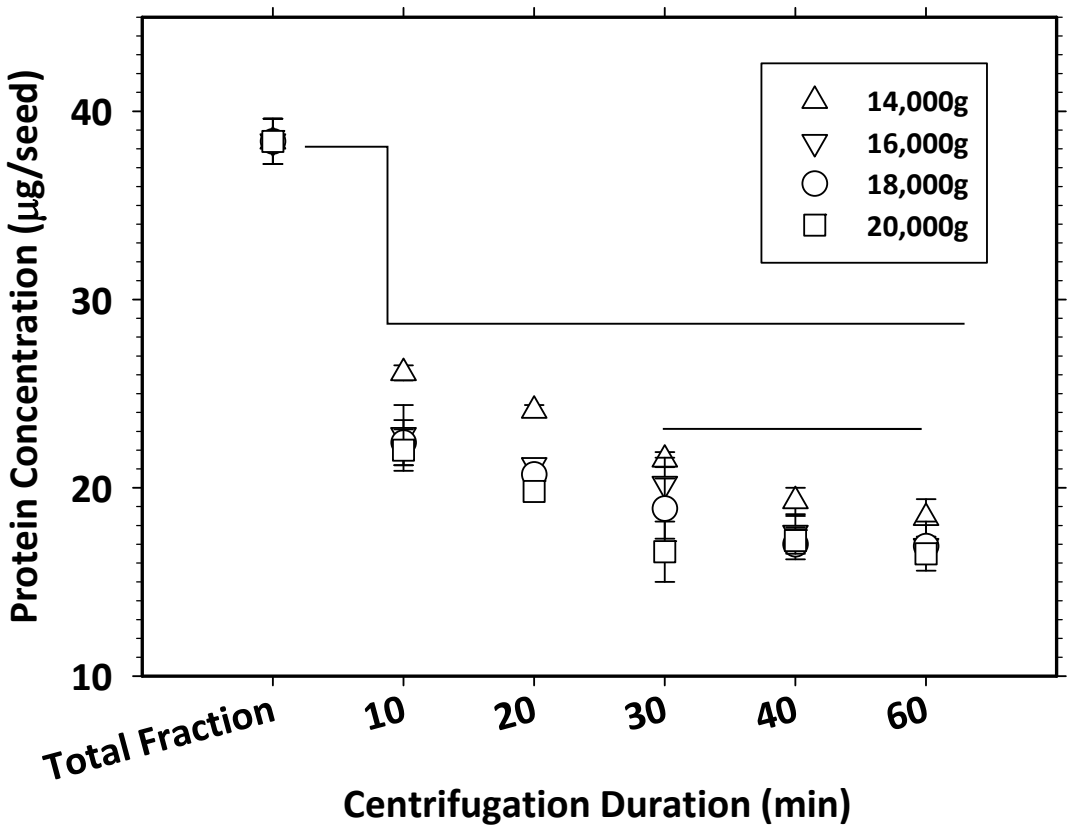


Figure 2.16. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for 7-day-flash dried and non-dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2°C [MC=14% (DWB), G=0%, V=5%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

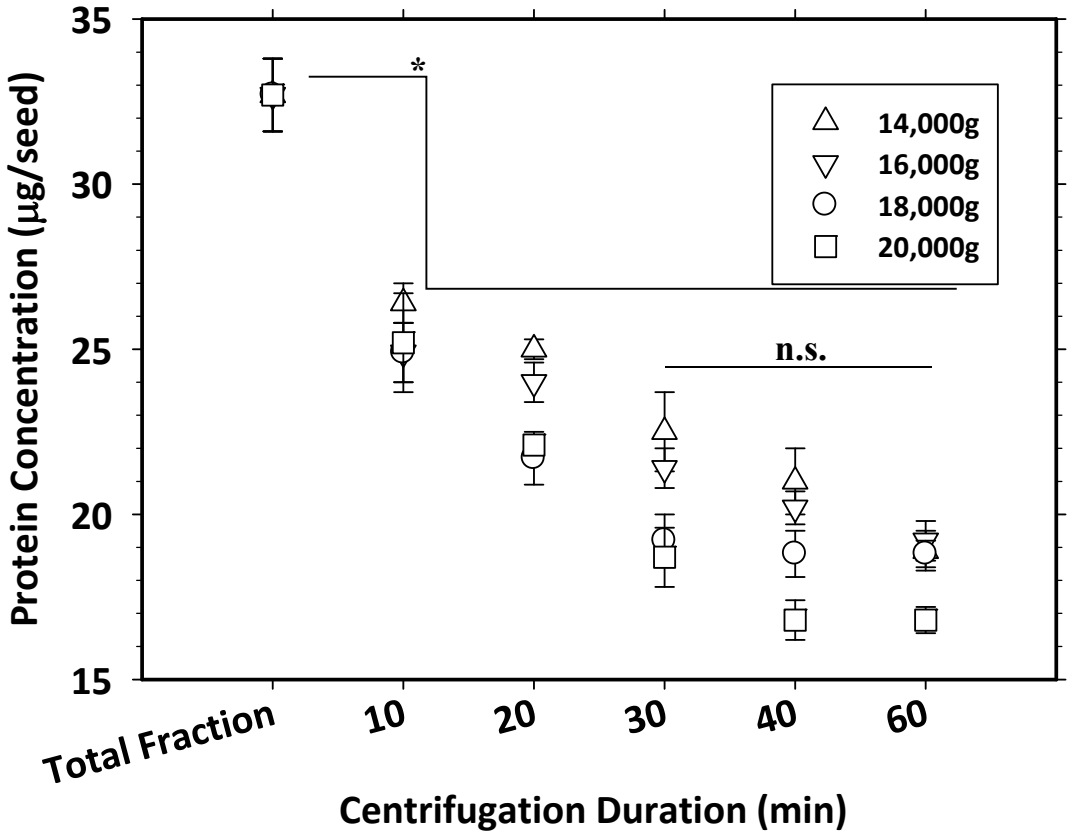


Figure 2.17. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for 7-day-flash dried and dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007), cold stratified for 1.5 months at 2^0C [MC=18% (DWB), G=0%, V=5%], flash frozen in liquid N_2 and stored at -80^{o}C for 6 months. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

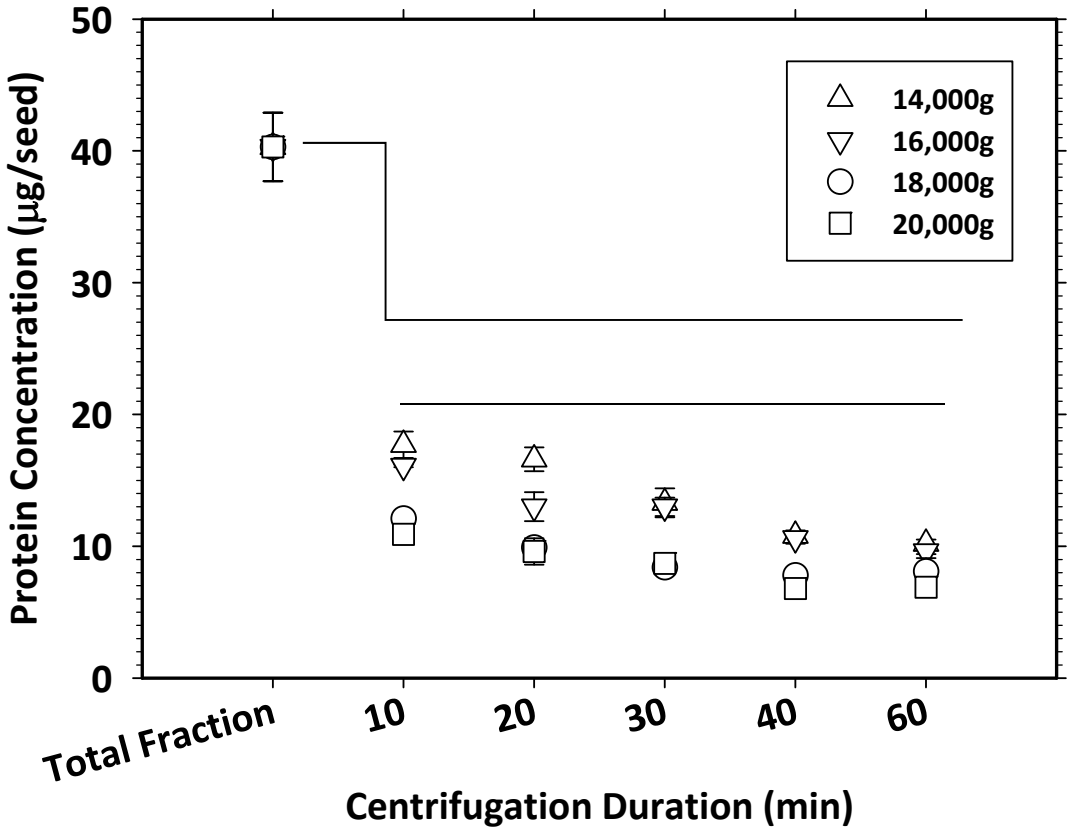


Figure 2.18. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for dry and aged *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and stored dry for 6 months at 23^{o}C [MC=9% (DWB), G=0%, V=0%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

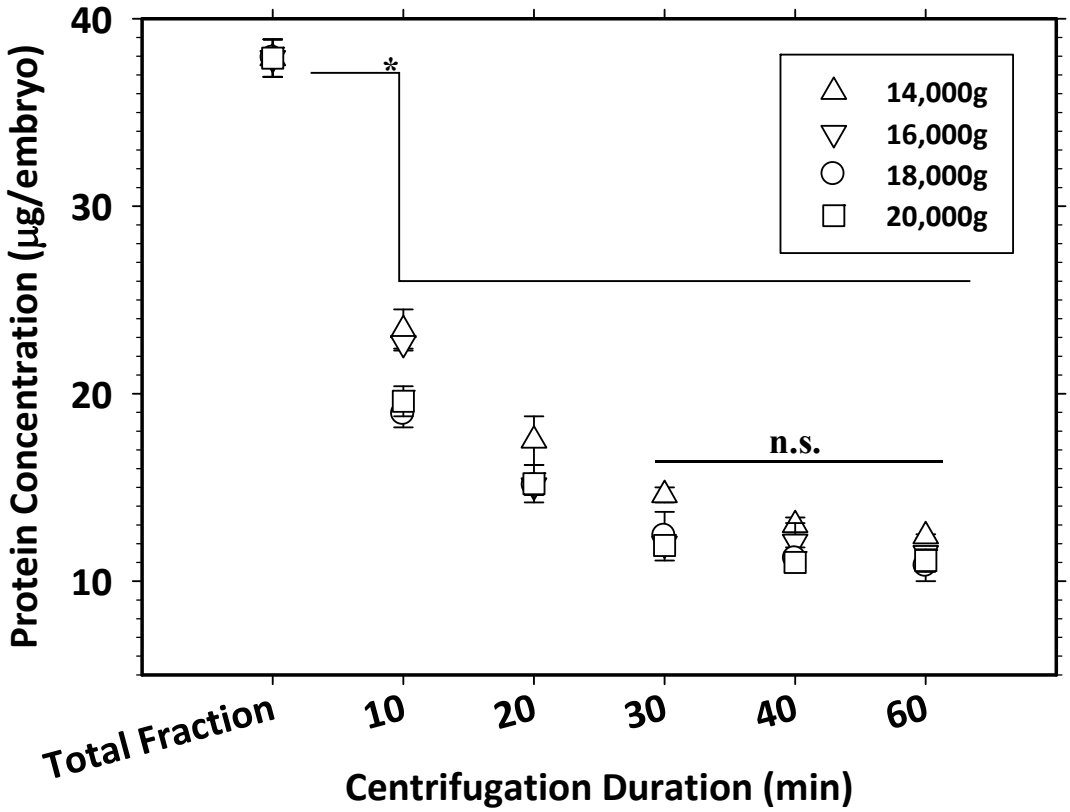


Figure 2.19. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for fully-hydrated and non-dormant *S. alterniflora* embryos. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007), cold stratified for 7 months at 2°C [MC (whole seed)=139%, G=90%, V=90%], flash frozen in liquid N_2 and cut to isolate the embryos. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

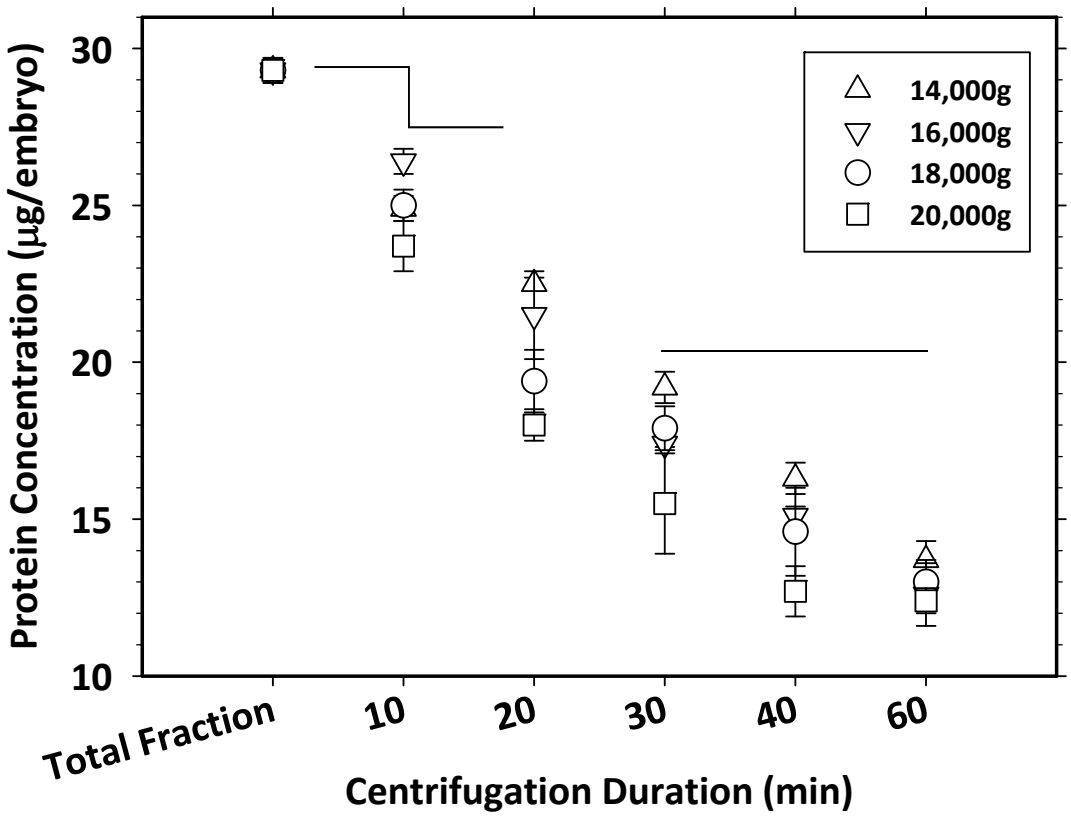


Figure 2.20. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for fully-hydrated and dormant *S. alterniflora* embryos. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007), cold stratified for 1 month at 2°C [MC (whole seed) =121% (DWB), G=0%, V=95%], flash frozen in liquid N_2 and cut to isolate the embryos. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

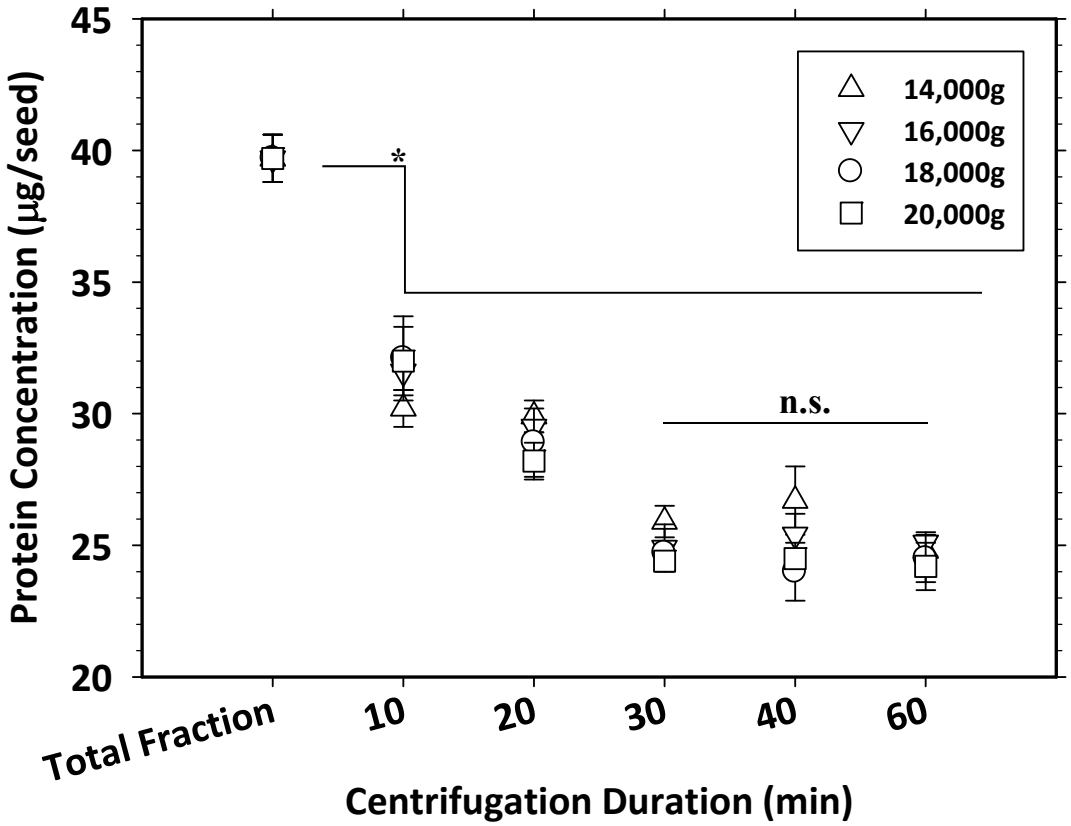


Figure 2.21. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for fully-hydrated and non-dormant *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), stored dry at -20^{o}C for 5 months, and cold stratified at 2^{0}C for 3 months [MC=114% (DWB), G=80%, V=85%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

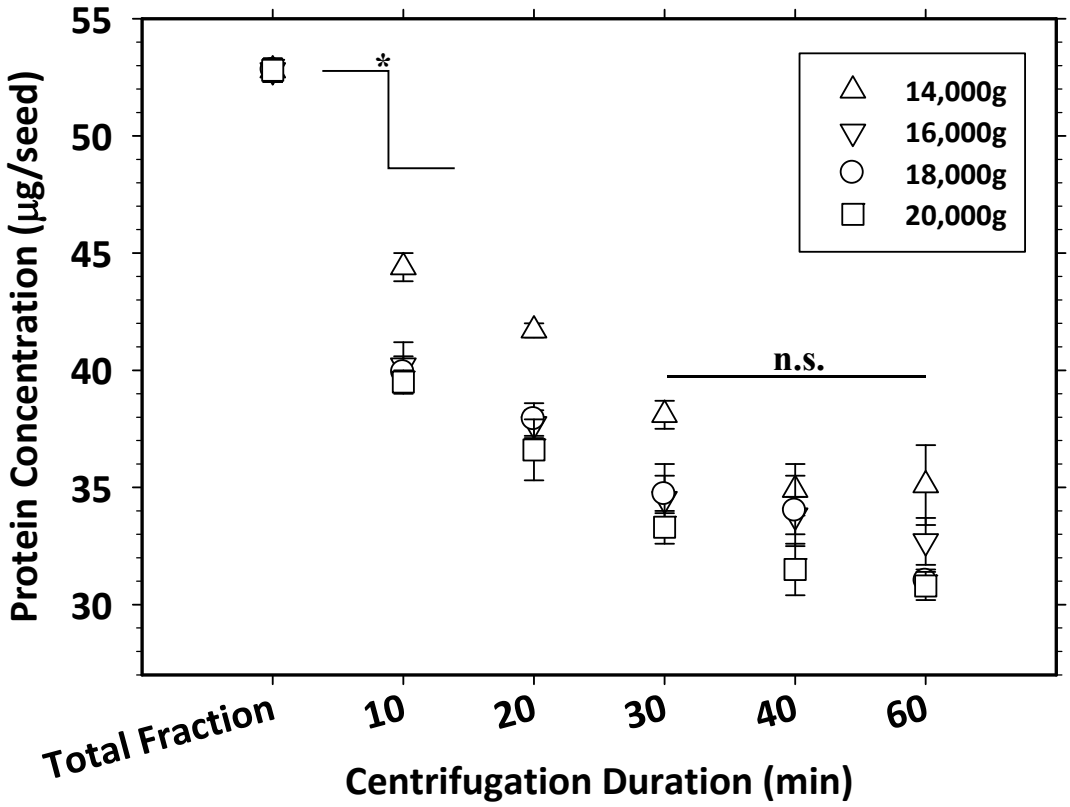


Figure 2.22. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for fully-hydrated and dormant *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), stored dry at -20^{o}C for 7 months, and cold stratified at 2^{0}C for 1 month [MC=143% (DWB), G=0%, V=85%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

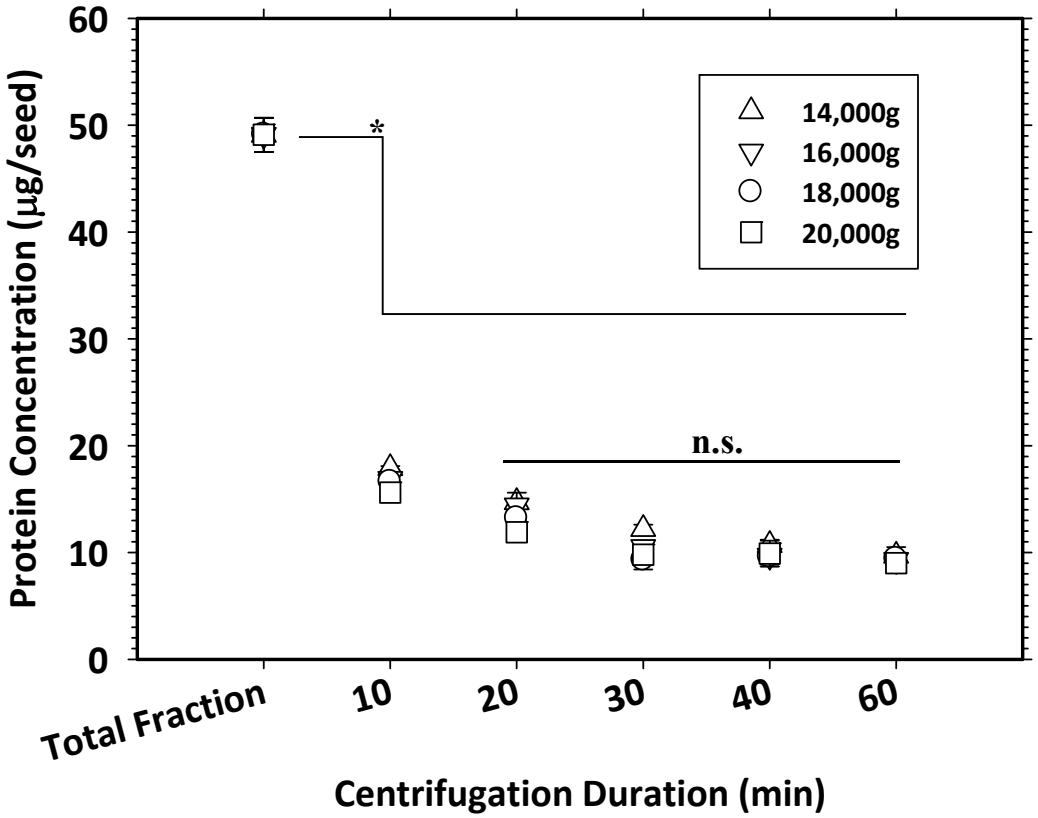


Figure 2.23. Protein concentrations of total fractions and heat-stable fractions prepared after different centrifugation speeds and durations for dry and dormant *S. pectinata* seeds. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20°C for 7 months [MC=10% (DWB), G=0%, V=88%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

The heat-stable fractions from radicles of *Glycine max* and *S. alterniflora* were significantly lower than those from seeds.

The Minimum Amount of Protease Inhibitor

The yields of total and heat-stable protein were not significantly different in extracts containing protease inhibitor (48.8 ± 1.8 and 28.2 ± 1.8 µg/seed for 50 µl; 48.2 ± 2.5 and 27.0 ± 1.8 µg/seed for 20 µl; 45.2 ± 0.88 and 26.4 ± 1.0 µg/seed for 10 µl) compared to the controls without protease inhibitor (44.7 ± 1.6 and 23.9 ± 1.6 µg/seed) ($p \geq 0.05$) (Fig. 2.25), and the differences were also not significantly different in comparisons among extracts with 10, 20 and 50 µl protease inhibitor. If proteolysis occurs without sufficient inhibitor, it is expected that proteome changes will be seen by electrophoresis. But there was no visual evidence of protein degradation in the 1-D gels in lanes of proteins containing 10 and 20 µl protease inhibitor; the low resolution of HS 50 µl is likely due to a loading problem (Fig. 2.26).

PDQ™ Protease Assay: for *S. alterniflora*, protease enzymatic activity was considerably reduced or completely suppressed by addition of 50ul of protease inhibitor (Table 2.8), except the heat-stable fraction in fully hydrated and dormant *S. alterniflora* (0.1811 BAEE/ml vs. 0.2066 BAEE/ml). In a comparison between dormant and non-dormant *S. alterniflora* or *pectinata*, protease activity in heat-stable protein fractions was higher in non-dormant seeds (1.2183 ± 0.8913 vs. 0.1811 BAEE/ml for *S. alterniflora*; 0.2080 vs. 0.0659 BAEE/ml for *S. pectinata*) (> ± 10%).

DISCUSSION

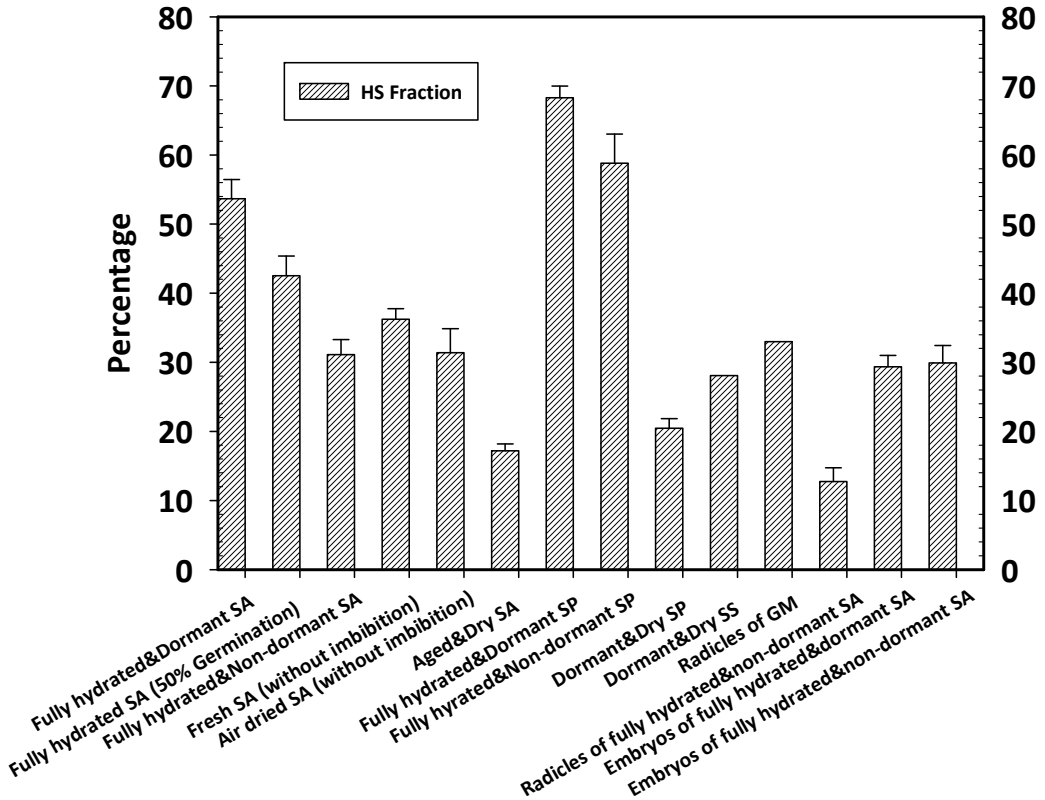
Optimum Heating Temperature and Duration

Heating at 95°C for at least 40 minutes is needed to obtain a heat-stable protein

Table 2.7. A summary of key values from Figures 2.24 (percentage of heat-stable protein fraction and desiccation tolerance). HS: heat-stable; [HS Fraction]: protein concentration of heat-stable fraction after heating at 95°C for 40 min, ice precipitation for 30 min and centrifugation at 20,000 g for 40 min. Each mean represents 3 biological replicates except for dry *S. spartinae** and radicles of *G. max** (1 biological replicate).

Species / Physiological States	*Spartina pectinata* (HS %)	*Spartina alterniflora* (HS %)	*Spartina spartinae* or *Glycine max* (HS %)
Fully hydrated & dormant	68 ± 2	54 ± 3	-
Fully hydrated (50% Germination)	-	43 ± 3	-
Fully Hydrated & non-dormant	59 ± 4	31 ± 2	-
Fresh harvested	-	36 ± 2	-
Air dried & fresh harvested	-	31 ± 3	-
Dry on plants	20 ± 1	-	*S. spartinae** 28
Radicles	-	13 ± 2	*G.max** 33
Fully hydrated & dormant embryos	-	29 ± 2	-
Fully hydrated & non-dormant embryos	-	30 ± 3	-

Figure 2.24. Percentages of heat-stable proteins in different systems. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) unless otherwise noted. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007). Fully-hydrated and dormant SA: cold stratified for 2 weeks at 2°C [MC=145% (DWB), G=0%, V=93%], flash frozen in liquid N_2 and stored at -80°C for 6 months. Fully-hydrated (50% germination): *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008) and cold stratified for 3 months at 2°C [MC=124% (DWB), G=45%, V=90%]. Fully-hydrated and non-dormant SA: cold stratified for 6 months at 2°C [MC=128% (DWB), G=93%, V=93%]. Fresh: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008) [MC=50% (DWB), G=0%, V=92%], flash frozen in liquid N_2 and stored at -80°C for 4 months. Air Dried: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008), air dried at 23°C for 3 weeks [MC=17% (DWB), G=0%, V=0%], flash frozen in liquid N_2 and stored at -80°C. Aged and dry SA: stored dry for 6 months at 23°C [MC=9% (DWB), G=0%, V=0%]. Fully-hydrated and non-dormant SP: stored dry at -20°C for 17 months, and cold stratified at 2°C for 3 months [MC=120% (DWB), G=85%, V=88%]. Dry and dormant SP: stored dry at -20°C for 7 months. [MC=10% (DWB), G=0%, V=88%]. Fully-hydrated and dormant SP: stored dry at -20°C for 20 months, and cold stratified at 2°C for 1 week [MC=139% (DWB), G=0%, V=85%]. Dry: *S. spartinae* seeds were harvested from Port Fourchon, LA (2008) [MC=6% (DWB), G=0%, V=60%], flash frozen in liquid N_2 and stored at -80°C. Radicles of fully-hydrated and non-dormant SA: cold stratified for 6 months at 2°C, and incubated at 24°C for germination. After 14-d germination, radicle emergences were carefully clipped by blade. Embryos of fully-hydrated and dormant: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008), cold stratified for 1 month at 2°C [MC (whole seed) = 121% (DWB), G=0%, V=95%], flash frozen in liquid N_2 and cut to isolate the embryos. Embryos of fully-hydrated and non-dormant: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2008), cold stratified for 7 months at 2°C [MC (whole seed)=139%, G=90%, V=95%], flash frozen in liquid N_2 and cut to isolate the embryos. Each mean represents 3 biological replicates except for dry *S. spartinae* and radicles of *G. max* (1 biological replicate). Error bars ± SE.


HS Fraction
Percentage
0
10
20
30
40
50
60
70
80
Fully hydrated&Dormant SA
Fully hydrated SA (50% Germination)
Fully hydrated&Non-dormant SA
Fresh SA (without imbibition)
Air dried SA (without imbibition)
Aged&Dry SA
Fully hydrated&Dormant SP
Fully hyrated&Non-dormant SP
Dormant&Dry SP
Dormant&Dry SS
Radicles of GM
Radicles of fully hydrated&non-dormant SA
Embryos of fully hydrated&dormant SA
Embryos of fully hydrated&non-dormant SA

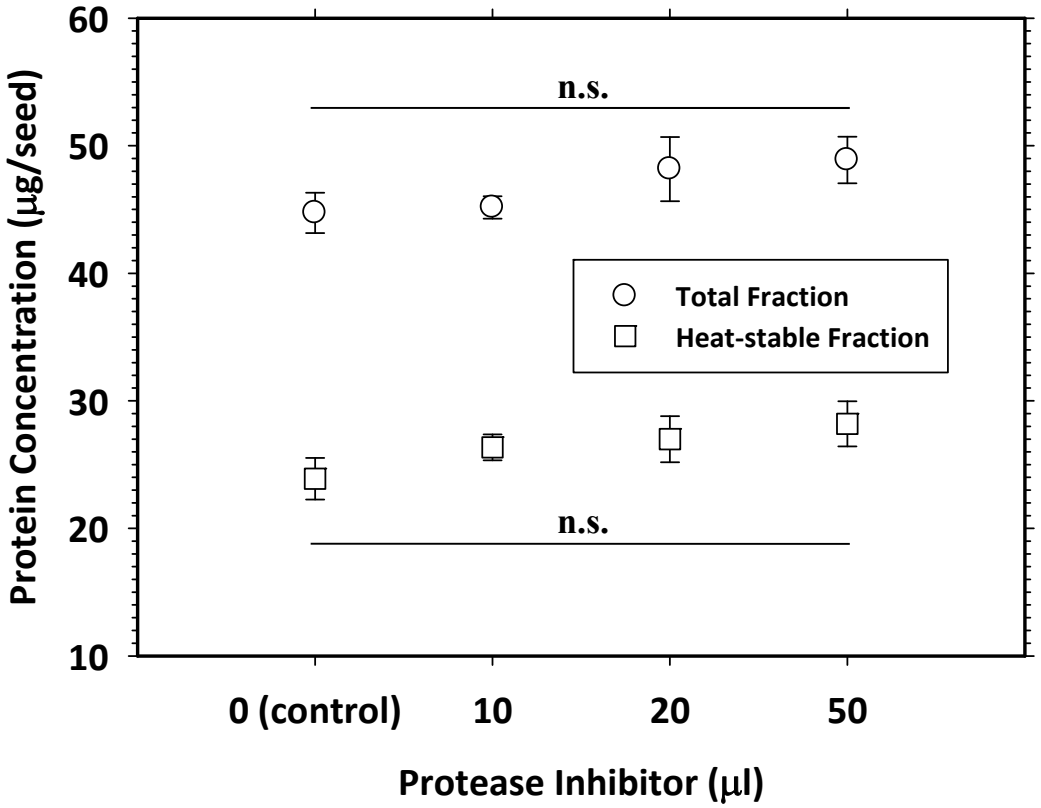


Figure 2.25. The effect of protease inhibitor on protein concentration of both the total and heat-stable fractions in the fully-hydrated and dormant *S. alterniflora* seeds. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 2 months at 2^{o}C [MC=111% (DWB), G=25%, V=100%]. Each mean represents 3 biological replicates. Error bars ± SE. *, significant; n.s., non-significant. Statistical significance was determined by one way analysis of variance (ANOVA) test of Holm-Sidak (α=0.05).

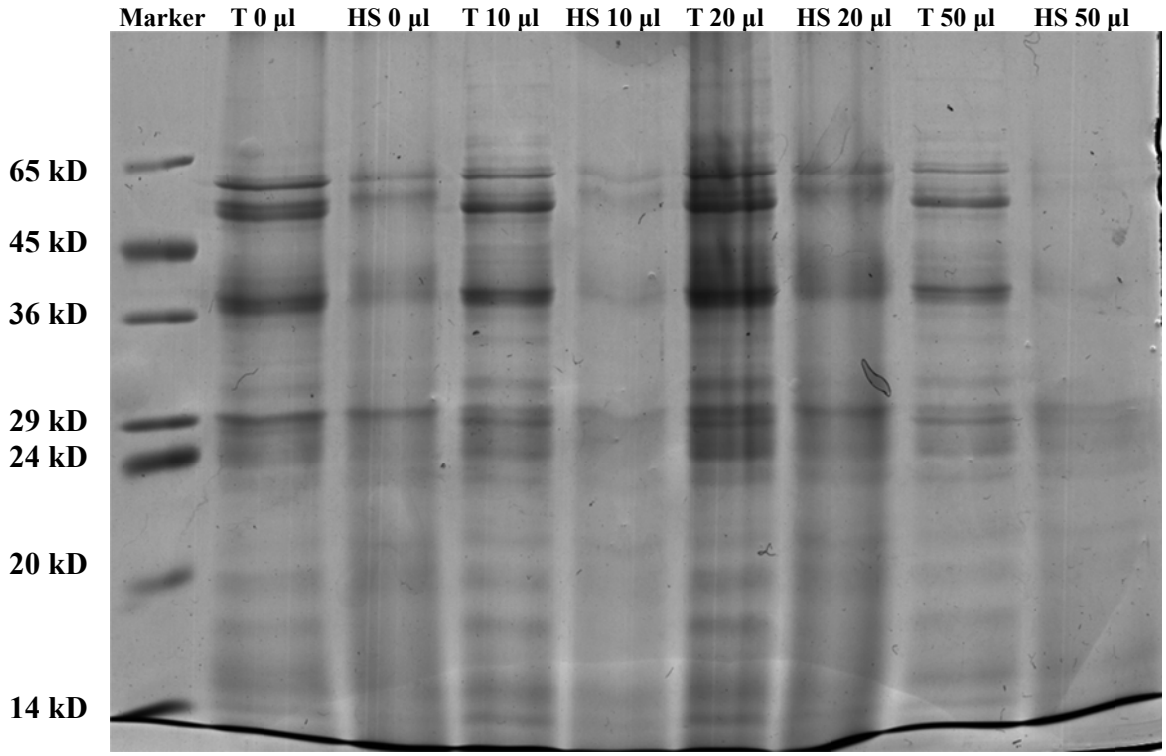


Figure 2.26. The effect of protease inhibitor amount (µl) on protein profiles of SDS-PAGE (12% polyacrylamide for resolving gel) in the fully-hydrated and dormant *S. alterniflora* seeds. T: total fraction; HS: heat-stable fraction. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 2 months at 2^{o}C [MC=111% (DWB), G=25%, V=100%]. Fifty micro-grams of proteins were loaded onto each lane.

Table 2.8. Comparisons of BAEE enzyme units per ml of PDQ™ colorimetric assay ± protease inhibitor. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007). Fully-hydrated and dormant: *S. alterniflora* seeds were cold stratified for 2 weeks at 2°C [MC=159% (DWB), G=0%, V=90%], flash frozen in liquid nitrogen and stored at -80°C for 6 months. Fully-hydrated & non-dormant: *S. alterniflora* seeds were cold stratified for 6 months at 2°C [MC=143% (DWB), G=90%, V=90%]. *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007). Fully-hydrated and dormant: *S. pectinata* seeds were stored dry at -20°C for 20 months, and cold stratified at 2°C for 1 week [MC=132% (DWB), G=0%, V=85%]. Fully-hydrated and non-dormant: *S. pectinata* seeds stored dry at -20°C for 17 months, and cold stratified at 2°C for 3 months [MC=126% (DWB), G=80%, V=80%]. Dry and dormant: *S. pectinata* seeds stored dry at -20°C for 7 months. [MC=8% (DWB), G=0%, V=85%]. For fully hydrated and non-dormant *S. alterniflora*, each mean represents 3 biological replicates. For others, 1 biological replicate was conducted. Absorbance was taken at 450 nm after a 3-hour reaction at 37°C. Error bars ± SE.

BAEE/ml / Samples	0 µl Protease Inhibitor	50 µl Protease Inhibitor
Fully Hydrated & Dormant SA Total Fraction	0.8148	0
Fully Hydrated & Dormant SA Heat-stable Fraction	0.1811	0.2066
Fully Hydrated & Non-dormant SA Total Fraction	2.3540 ± 1.1435	0.0907 ± 0.1571
Fully Hydrated & Non-dormant SA Heat-stable Fraction	1.2183 ± 0.8913	0.1425 ± 0.1553
Fully Hydrated & Dormant SP Total Fraction	0.2880	0.1190
Fully Hydrated & Dormant SP Heat-stable Fraction	0.0659	0
Fully Hydrated & Non-dormant SP Total Fraction	0.3151	0
Fully Hydrated & Non-dormant SP Heat-stable Fraction	0.2080	0.1680
Dry & Dormant SP Total Fraction	0.2610	0.0360
Dry & Dormant SP Heat-stable Fraction	0.1748	0

fraction from the *S. alterniflora*, *S. pectinata* and *S. spartinae* seeds. A rapid protein precipitation was observed consistently during the first 10 min of heating (Figs 2.2-2.11). The speed of protein precipitation gradually slowed down after 10 minutes, and reached a constant level by 40 min at 75^{o}C, 85^{o}C and 95^{o}C (Figs 2.2-2.11). A continued heating after 40 min did not significantly denature more soluble protein at these three temperatures.

For the total fraction, protein concentration was slightly higher in the dormant than non-dormant fully hydrated *S. alterniflora* (53.8 ± 1 μg/seed vs. 49.2 ± 0.9 μg/seed) ($p=0.027<0.05$, Student's *t*-test) and *S. pectinata* (56.3 ±0.5 μg/seed vs. 51.2 ± 0.7 μg/seed) ($p=0.004<0.05$) seeds (Table 2.4). In comparisons of both Fig. 2.1 vs. Fig. 2.2 and Fig. 2.7 vs. Fig. 2.8, a smaller heat-stable fraction of soluble protein in non-dormant seeds was observed. A similar downward trend of protein concentration during cold stratification was observed in the recalcitrant horse chestnut *Aesculus hyppocastanum* seed (Gumilevskaya *et al.*, 2001).

One possible explanation is mobilization of storage proteins during moist chilling. The non-dormant seeds have been submerged in water for months, and metabolism for preparation for seed germination is taking place. So, a smaller amount of storage proteins (non-dormant *S. alterniflora* seeds are able to germinate at 2^{o}C when cold stratified for 10 months) could explain a lower fraction of heat stable proteins in non-dormant *S. pectinata* and *S. alterniflora* seeds. A lower heat-stable fraction correlated with fewer storage proteins in comparisons of Figs 2.2, 2.3 and 2.7, in which the fractions of heat-stable proteins are much lower in the embryo than in the whole seed (25%, 26% vs. 58%, 78%). In addition, in isolated embryos of *S. alterniflora*, heat-stable soluble proteins

reached a constant level after 20 min heating at 95°C (compared to heating at 95°C for 40 min in the whole seed), which could also be explained by fewer heat-stable proteins in the embryo. It is also possible that the heat stable proteins in the embryo are less heat stable than those in the endosperm.

A comparison between isolated dormant and non-dormant *S. alterniflora* embryos (Fig. 2.7; Table 2.4) shows almost the same total protein amounts (36.0 ± 0.8 µg/embryo vs. 33.6 ± 1.2 µg/embryo) ($p=0.171>0.05$), as well as for the 40-min heated fractions from D vs. ND embryos (9.1 ± 0.9 µg/embryo vs. 8.6 ± 0.3 µg/embryo) ($p=0.626>0.05$), which indicates that the changes with stratification were centered on endosperm proteins during whole seed stratification. However, comparisons of Fig. 2.2, 2.3 and 2.7 suggest that the endosperm protein concentration of 40-min heat-stable fraction is even a bit higher than the total fraction in dormant and non-dormant *S. alterniflora*, which is possibly due to the use of seeds that were harvested from two different years (2007 and 2008). Another possible explanation is that proteins that remain soluble after heating treatment may preferably react with the Bradford reagent, so that the assay will give a higher detected value. The Coomassie Brilliant Blue G-250 stain of the Bradford reagent has a favorable binding to basic and aromatic amino acids (Compton and Jones, 1985); therefore, the intensity of colorimetric detection of the heat-stable proteins is greatly dependent on the their composition of amino acids.

Artificial drying reduced the amount of extractable soluble protein in *S. alterniflora* (Figs. 2.4-2.6; Table 2.4). The protein concentrations of the total and heat-stable fractions was higher in fully hydrated (49.2 and 28.7 µg/seed for ND, 53.8 and 39.8 µg/seed for D) than either flash dried or aged *S. alterniflora* (36.8 and 14.8 µg/seed

for ND, 37.8 and 17.1 μg/seed for D, 34.0 and 9.1 μg/seed for aged). A likely explanation is that extraction from dry material is less efficient than hydrated material. Another possible explanation for the decreased protein concentration during desiccation is the occurrence of protein denaturation and precipitation that might occur with death of these recalcitrant seeds. Severe desiccation, shrinking the cellular volume and crowding the cytoplasmic components increases the molecular interactions, which can cause protein denaturation (Hoekstra *et al.*, 2001).

The difference of protein concentration between hydrated dormant and non-dormant *S. alterniflora* mentioned above, however, was not observed in flash dried seeds (36.8 ± 1.1 vs. 37.8 ± 0.4) ($p=0.441>0.05$) (Figs 2.4 and 2.5). A likely explanation is that the seeds were harvested from different years, 2007 and 2008 (Figs 2.4 and 2.5). The decreased percentages of proteins during flash drying were not the same in a comparison between dormant (30.2%) and non-dormant (25.2%) *S. alterniflora*, indicating that some proteins in dormant seeds may be more sensitive in response to flash drying.

In *S. pectinata*, more protein in the total fraction was also observed in rehydrated than dry and dormant (1^{st} dry) seeds (Figs 2.8, 2.9 and 2.10). If drying can affect protein concentration, it is expected that the protein concentration of total fraction in flash dried *S. pectinata* (2^{nd} dry) will drop to the same level as the dry and dormant (1^{st} dry) seeds. This assumption will be determined in the future experiments.

Significant changes of heat resistance of proteins have been observed to be associated with different physiological states (desiccation tolerance and dormancy) of *Arabidopsis* seeds (Wolkers *et al.*, 1998), in which proteins from desiccation tolerant and dormant *Arabidopsis* wild types are more heating resistant than proteins from desiccation

sensitive and non-dormant single mutants (*lec1-1*, *lec1-3*, *abi3-5*) and double mutants of *aba1-1* and *abi3-1*. For *Spartina* seeds, in contrast, while proteins seem more resistant to heat denaturation at 75°C and 85°C than at 95°C in hydrated desiccation tolerant and non-dormant *S. pectinata* (Fig. 2.8), such differential heat sensitivity was not obvious in hydrated desiccation tolerant and dormant seeds (Fig. 2.9), nor in any of the *S. alterniflora* samples (Figs 2.2-2.3).

The variances of protein concentration between dormant or non-dormant, or between fully hydrated and dried seeds, also might have been caused by the changes of the sensitivity of the Bradford reagent during storage in the refrigerator. A reagent with lower sensitivity might result from several months' storage of the reagent compared to when the bottle is newly opened. However, no significant difference of detection sensitivity of the Bradford reagent has been observed (Fig. A-4). The effective chemical of the Bradford reagent is Coomassie Brilliant Blue G-250, which is dissolved in phosphoric acid at room temperature. During the cold storage, the Coomassie Brilliant Blue can precipitate, and its concentration could change. To make sure that Coomassie Brilliant Blue is completely dissolved, the reagent bottle was inverted several times and was not used in my experiments until the reagent reached the room temperature.

Because 100°C was used in several other investigations to obtain a heat-stable fraction (Table 2.1), the difference between 95°C and 100°C was determined for *S. alterniflora* protein extracts. The protein concentrations of 40-min heat-stable fraction at 95°C and 100°C were the same (Table A-1), confirming that heating at 95°C for 40 min is sufficient to obtain a heat-stable protein fraction.

Optimum Duration for Cold Precipitation

Cold precipitation for 30 minutes was sufficient to precipitate the heat-unstable protein in both the *S. pectinata* and *S. alterniflora* seeds, but no significant difference of heat-stable protein concentration was observed in comparisons among room temperature, 0, 10, 20 and 30 minutes of ice incubation conditions in either *S. alterniflora* or *S. pectinata* (Figs 2.12 and 2.13). The 30 min cold precipitation was selected for several reasons: (1) cold incubation of protein extracts for 30 min provides sufficient time for tubes to cool down; (2) the optimum temperature for proteases generally ranges from 35-45^{o}C, and the protease activities have been observed in protein extracts of either *S. alterniflora* or *S. pectinata* at 37^{o}C (Table 2.8); therefore, a longer duration of cold incubation, letting the protein extracts cool down, may minimize the protein degradation caused by proteases; (3) the 30 min ice incubation provides time for preparation of downstream aspects of the experimental protocol.

For *S. alterniflora*, consistent with Figs 2.2 and 2.3, protein concentration was higher in dormant than non-dormant *S. alterniflora* for the total (57.5 µg/seed vs. 52.9 µg/seed) ($p \leqslant 0.001$) and heat-stable (42.8 µg/seed vs. 31.2 µg/seed) ($p \leqslant 0.001$) fractions (Fig 2.12). The amount of heat stable proteins recovered after the 30 min ice precipitation time was non-significant ($p \leq 0.05$) compared to the values obtained under the same conditions as in Figs 2.2-2.7, with the exception of the embryo HS fraction; this difference could be due to embryo variation between the two seed harvest years used, 2008 (Fig. 2.7) and 2007 (Fig. 2.12).

For *S. pectinata* HS fractions from hydrated seeds (Figs 2.8-2.10 versus Fig. 2.13), the results are more problematic: in Fig 2.13 the HS protein fraction averaged 12 µg/seed

after the 30 min ice incubation, but ranged from 18 to 38 µg/seed in Figs 2.8-2.10. Seeds used in Figs 2.8-2.10 had been stored unimbibed 5-7 months at -20°C before initiation of the experiments, while *S. pectinata* seeds in Fig 2.13 had been stored at -20°C for as long as 20 months. During this extended period of dry storage, viability declined from 91% to 86%. While the precise reasons for the quantity of the heat stable fraction cannot be identified, these data suggest the need to use *S. pectinata* seeds with comparable viabilities for accurate comparisons of proteomic profiles.

Optimum Centrifugation Speed and Duration

A centrifugation speed of 20,000 g for 40 minutes is needed to obtain the heat-stable fraction in both the *S. pectinata* and *S. alterniflora* seed/embryo.

Figs 2.2-2.11 are only comparable to Figs 2.14-2.23 of centrifugation at 14, 000 g and 40 minutes, because they were used for optimization of heating temperature and duration in Figs. 2.2-2.11. In a comparison of non-dormant *S. alterniflora* between Figs. 2.2 and 2.14, the protein concentration in the 14,000 g, 95^{o}C protein fraction is lower in Fig. 2.14 (22.6 µg/seed) than in Fig. 2.2 (28.7 µg/seed) ($p \leq 0.001$). For dormant *S. alterniflora*, the total fraction was more in Fig. 2.15 (61.5 µg/seed) than Fig. 2.3 (53.8 µg/seed), but HS fraction was less in Fig. 2.15 (31.8 µg/seed) than Fig. 2.3 (39.8 µg/seed) ($p \leq 0.001$). For dried *S. alterniflora*, the total and HS fractions are non-significantly different comparing between Figs 2.4-2.6 and Figs 2.16-2.18, except the HS fraction of flash dried and non-dormant *S. alterniflora*. For isolated *S. alterniflora* embryos, the total fraction was not significantly different (Fig. 2.7 vs. Figs 2.19-2.20), but the HS fraction was higher in Figs. 2.19-2.20 (12.4 µg/seed and 13.7 µg/seed) than Fig. 2.7 (9.1 µg/seed and 8.6 µg/seed) ($p \leq 0.001$). The difference of HS fraction may be caused by the use of *S.*

alterniflora seeds that were harvested in different years (2007 vs. 2008). For the same year harvested seed (Fig. 2.12 vs. Figs 2.19-2.20), the total and HS fractions were not significantly different, except the HS fraction of non-dormant *S. alterniflora*.

For *S. pectinata*, there was no significant difference in protein concentration of total and HS fractions observed (Figs. 2.8-2.10 vs. Fig. 2.21-2.23), except the total fraction of hydrated and non-dormant and HS fraction of dry *S. pectinata*. The different cold-stratification times and viability percentage (Figs. 2.8-2.9 vs. Figs. 2.21-2.22) may also affect the protein concentration.

Percentage of Heat-Stable Protein Fractions and Desiccation Tolerance

It is concluded from Fig. 2.24 that there is no clear association between the percentage of heat-stable protein fractions and the degree of desiccation tolerance, and the percentage of heat-stable protein fractions is determined by the physiological states of seeds. Desiccation tolerant *S. pectinata* has more heat-stable proteins than desiccation intolerant *S. alterniflora* in fully hydrated, dormant and non-dormant seeds; however, desiccation tolerant dry and dormant *S. pectinata* has a lower heat-stable fraction than *S. alterniflora* of any physiological states. Heat-stable fractions of dormant seeds were significantly higher than non-dormant seeds in both fully hydrated *S. alterniflora* and *S. pectinata*. Heat-stable fractions of fully hydrated *S. alterniflora* and *S. pectinata* seeds were significantly higher than dry seeds. The whole seeds of *S. alterniflora* have a higher heat-stable fraction than embryos. The heat-stable fractions of radicles of *Glycine max* and *S. alterniflora* were significantly lower than seeds.

Orthodox seeds contain LEA proteins, and these LEAs disappear during or shortly after visible germination (radicle protrusion, in most cases) (Boudet *et al.*, 2006),

coinciding with a loss of seedling desiccation tolerance. On the basis of this common observation, heat-stable LEA proteins have been suggested to be associated with desiccation tolerance. For *S. alterniflora*, soluble heat stable proteins are significantly higher in hydrated and dormant (Fig. 2.3) than in dried seeds (Fig. 2.5), which could suggest that a reduction of heat stable fraction during desiccation may cause recalcitrant seed death. However, the hypothesis is not supported by the following results. (1) If desiccation tolerance is related with a higher HS protein fraction, it is expected that HS protein fraction is higher in orthodox than in recalcitrant seeds. However, air dried *S. alterniflora* (without rehydration) has a higher heat-stable fraction than dry *S. pectinata* (without rehydration) (Fig. 2.24, Table 2.7). (2) Heat resistant proteins decrease during the transition from the dormant to non-dormant state in both *S. alterniflora* and *S. pectinata* seeds. In *S. alterniflora*, soluble heat-stable proteins decrease from completely dormant seeds to a seed population that is 50% dormant, and are fewest in completely non-dormant, ungerminated seeds. Therefore, the higher fraction is more likely to be associated with dormancy breaking (Fig. 2.24, Table 2.7). (3) The conclusion above is based on the assumptions that LEAs are related with desiccation tolerance, and the HS protein fractions in *Spartina* contain LEAs. However, no evidence has been obtained to support these assumptions at this stage of the research.

Decreased HS protein fractions have been observed in *S. alterniflora* and *S. pectinata* during cold stratification (Fig. 2.24). The HS protein fraction is significantly lower in germinated *S. alterniflora* seedlings than ungerminated *S. alterniflora* seeds (Fig. 2.24). A similar trend was also reported in a study of recalcitrant *A. hippocastanum* (Gumilevskaya *et al.*, 2001), in which HS protein was reduced during cold stratification

and germination. Since mobilization of storage protein in endosperm occurs during germination, it is implicated that storage proteins may explain the decreased HS proteins observed during *S. alterniflora* cold stratification (Fig. 2.24), especially since some storage proteins are reported as soluble and heat-stable (González-Pérez *et al.*, 2005; Oliveira *et al.*, 2007). *S. alterniflora* is able to remain in an ungerminated state at 2^{o}C when cold stratified for 6-8 months, and mobilization of storage proteins might be taking place in preparation for germination. Thus, storage proteins in non-dormant *S. alterniflora* that has been cold stratified for 6 months should be less than dormant seeds.

Interestingly, rehydration was found to increase the fractions of heat-stable proteins in both *Spartina* species (Fig. 2.24). It is lower in freshly harvested than rehydrated *S. alterniflora*. In *S. pectinata*, dry and dormant seeds yield a much lower heat-stable fraction. The results seem to be inconsistent with the assumption that drying induces the expression of heat-stable LEA proteins that help seed to survive desiccation. A possible explanation is that rehydration, like cold stratification and germination, may affect the storage proteins. Long-term imbibition may help the water-soluble storage protein, e.g. albumin, better dissolved in water; thus rehydrated seeds yield a higher heat-stable fraction. In dry seeds, although the protein extraction buffer is able to dissolve storage proteins such as albumin, globulin and glutelin, 15 minutes of hydration during protein extraction may not be enough to dissolve all of them. Therefore, the effect of a prolonged protein extraction on the yield of the heat-stable fraction was tested. However, the crude extract incubated at 4^{o}C for a day failed to yield a higher fraction of heat-stable proteins than the control in the dry seed of both *S. alterniflora* (9.2 vs. 9.3 µg/seed) and *S. pectinata* (15.1 vs. 15.4 µg/seed).

HS protein fractions are comparable between desiccation sensitive *S. alterniflora* embryos (29% ± 2) (Table 2.7) and desiccation tolerant *M. truncatula* embryos (28%) (Boudet *et al.*, 2006). The HS protein fraction in desiccation sensitive *S. alterniflora* radicles (13% ± 2) (Table 2.7) is comparable to that in desiccation sensitive *M. truncatula* (19%) (Boudet *et al.*, 2006).

In a summary, the heat-stable fraction percentage does not seem to be a useful indicator to gauge the extent of desiccation tolerance.

The Minimum Amount of Protease Inhibitor

The yields of total and heat-stable protein were not significantly different in extracts containing protease inhibitor (Fig. 2.25), and there was no visual evidence of protein degradation in the 1-D gels (Fig. 2.26). By using PDQ^{TM} Protease Assay, protease enzymatic activity was considerably reduced or completely suppressed by addition of 50µl of protease inhibitor in *S. alterniflora* and *S. pectinata* (Table 2.8).

Protein concentration of extracts with various amounts of protease inhibitor added was not significantly different. The effect of various amounts of protease inhibitor on profiles of one-dimensional gels was neither obvious. More proteins of low molecular weight would be evident. But the major protein bands, which are 63 kDa, 60 kDa, 36 kDa, and 29 kDa, consistently appeared independent of the amount of protease inhibitor. More proteins bands of low molecular weight were also not observed. However, a tiny amount of proteases often escapes detection by one-dimensional SDS-PAGE (White *et al.*, 1993). Therefore, a more sensitive and accurate method should be used to test the minimum amount of protease inhibitor added to extraction buffer to completely stop the protease activity.

For *S. alterniflora*, protease enzymatic activity was considerably reduced or completely suppressed (Table 2.8), except the heat-stable fraction in fully hydrated and dormant *S. alterniflora* (0.1811 BAEE/ml vs. 0.2066 BAEE/ml). One possibility is that some proteases in protein extract are heating activated. However, this assumption was not further confirmed in non-dormant seeds, in which the value of protease activity of HS fraction was not significantly different from the total fraction (0.1425 ± 0.1571 BAEE/ml vs. 0.0907 ± 0.1553 BAEE/ml) ($p=0.826>0.05$). A standard curve of the PDQ™ assay is shown in Appendix A-5.

Consistent with *S. alterniflora*, proteolytic activity was also completely suppressed in *S. pectinata*, except the total fraction of dormant and HS fraction of non-dormant seeds (Table 2.8). Proteolytic activity of HS fraction is lower than total fraction in hydrated dormant and dry dormant seeds (Table 2.8); but based on the standard error values for replicated samples, such putative incomplete suppression, as in *S. alterniflora*, can be rationalized as within the statistical precision of the assay.

In a comparison between dormant and non-dormant *S. alterniflora* and *pectinata*, protease activity was higher in non-dormant seeds (2.3540 BAEE/ml vs. 0.8148 BAEE/ml; 0.3151 BAEE/ml vs. 0.2880 BAEE/ml). The result was expected because metabolism should be more active in non-dormant than dormant seeds. Higher protease activity in non-dormant seeds suggests the active role of protease in breaking down seed storage proteins for germinating seeds.

While addition of 20 µl protease inhibitor was suggested for 0.1 gram seed extracts in 5 ml extraction buffer (Sigma-Aldrich), 50 µl protease inhibitor (1% v/v), two and half times of the suggested volume, was only tested. Further excess of protease

inhibitor may covalently change the proteins in extracts and cause artifactual spots on electrophoretic gels (Görg *et al.*, 2004), and so more than 50 µl protease inhibitor was not tested.

The temperature during seed protein extraction (4^oC) was different from that for protease assay (37^0C). Since the final purpose is to determine the proteolysis during protein extraction, it seems necessary to measure the protease activity by using the protease assay at 4^0C. However, because proteolytic activity is inversely related with temperature (Görg *et al.*, 2004), it is expected that the effect of protease activity on protein extracts would be negligible at 4^oC if it is low at 37^oC.

In a summary, 50 µl protease inhibitor seems sufficient for protein extracts in *S. alterniflora* and *S. pectinata*.

SUMMARY

An optimized protocol for heat-stable protein extraction in *S. pectinata*, *S. spartinae* and *S. alterniflora* was obtained as follows: ground seed tissues (in liquid nitrogen) were transferred to a glass homogenizer and homogenized with 3 ml of extraction buffer (50mM HEPES, pH 7.5) (ice cold) and 50 µl of protease inhibitor (stored at -20^0C) added. Protein extract was transferred to a clean plastic tube (15ml) and the homogenizer was washed with an extra 2 ml of HEPES buffer (pH 7.5). The protein extract was centrifuged at 14,000g at 4°C for 20 min, twice. The protein supernatant after centrifugation was heated at 95^oC for 40 min and ice-incubated for 30 min. Then, heat denatured proteins was spun down and removed by centrifuging at 20,000 g for 40 min.

CHAPTER 3

IDENTIFICATION OF HEAT-STABLE PROTEINS THAT MAY BE ASSOCIATED WITH RECALCITRANCE BY USING ONE-DIMENSIONAL GEL ELECTROPHORESIS

INTRODUCTION

Since results of proteomics could be affected by many factors, such as technique, apparatus quality, staining method, variance in different protein samples, *etc.*, it is very important to obtain reproducible protein profiles. SDS-PAGE profiles of different sets of *Spartina* seed samples were evaluated to confirm the reproducibility of the protein profiles using the procedures optimized in Chapter 2. SDS-PAGE was used to compare the profiles of heat stable seed proteins between recalcitrant and orthodox *Spartina* species. SDS-PAGE is a one-dimensional protein separation based on protein molecular weight, because intrinsic ion charges of proteins were negligible compared to anionic charge of SDS, as SDS binds to polypeptides in a constant ratio of 1.4 g/g of polypeptide (Weber and Osborn, 1969).

METHODS

Seed Materials

S. alterniflora seeds were harvested in November, 2006-2008 from marshes of Port Fourchon, Louisiana. Seeds were collected by hand shattering. Seeds were immediately sealed in plastic zipper bags when harvested and put in a cool place after harvest. After transportation to laboratory, ten gram aliquots of seeds were put in Magenta vessels (Sigma Aldrich, St. Louis, USA) with 250 ml of deionized water. These seeds were placed at 2^{O}C and could be stored for up to 8-10 months. For aged *S. alterniflora* seeds, fresh harvested seeds were stored and dried in zipper bags at 2^{o}C.

Dried *S. pectinata* seeds were purchased from Western Native Seed (Coaldale, CO, USA), harvested in 2007 and mailed to the laboratory (Louisiana State Univ., Baton Rouge, LA., USA). After the seeds arrived at the laboratory, they were immediately stored dry in Mason jars at -20^{o}C. For wet *S. pectinata* seeds, ten grams of seeds were put in Magenta vessels with 250 ml of deionized water and stored at 2°C.

Extraction of Total Soluble and Heat-stable Proteins

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., St. Louis, USA) unless stated. Fifty seeds (0.1 g dry weight) of *S. alterniflora or S. pectinata* were freeze-clamped in liquid nitrogen, and then ground with a pestle in a mortar that was buried in dry ice. Ground powders were immediately transferred into a pre-cooled glass homogenizer, 50 µl of protease inhibitor (1% v/v) and 3 ml (*S. alterniflora and S. pectinata*) of extraction buffer {50mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, pH 7.5} (stored at 4^{o}C) were added into the homogenizer. The mixture was fully homogenized at 4^{o}C by 30 pestle strokes, and then transferred into a 10 ml clean plastic tube (Corning, Corning Co., Lowell, MA, USA) that was pre-cooled on ice. The homogenizer was washed with 2 ml of extraction buffer (*S. alterniflora and S. pectinata*), ensuring that the protein extracts were completely transferred. The total homogenate was centrifuged at 14,000g, at 4^{o}C for 20 minutes. After centrifugation, supernatant was carefully transferred into another clean plastic tube with a Pasteur pipette (VWR International), and then centrifuged again at 14,000g, at 4^{o}C for 20 minutes. The final supernatant was carefully pipetted to a clean plastic tube and divided into two portions. One was used for the concentration determination by the Bradford method (Bradford, 1976) and total protein preparation. The other was used for

heat-stable protein preparation; the supernatant was incubated at 95^{o}C for 40 minutes. After heating, the tubes were buried in ice for 30 minutes, and then centrifuged at 20,000g, at 4^{o}C for 40 minutes. Each supernatant was carefully pipetted out with Pasteur pipettes into clean plastic tubes, and protein concentration was measured by Bradford procedure (Bradford, 1976).

Electrophoresis

TCA (trichloroacetic acid) (100%) was added to solutions of either the total or heat-stable fraction to make a final concentration of 10% TCA (w/v). The mixture was buried in ice overnight (*ca.* 12 hours). The cold-incubated mixture was centrifuged at 16,000g at 4^{o}C for 30 minutes. The supernatant was carefully decanted after centrifugation. The pellet was fully washed by cold acetone (-20^{o}C) and centrifuged at 16,000g, at 4^{o}C for 10 minutes. The supernatant was carefully pipetted and discarded. The washing process was repeated three times. The pellet was vacuum dried (Speed Vac, SAVANT) and then dissolved in 100 µl of rehydration solution [7M urea (Bio-Rad Laboratories, USA), 2M thiourea (Bio-Rad Laboratories, USA), 4% CHAPS (w/v) (Bio-Rad Laboratories, USA) and 20mM dithiothreitol (DTT) (GE Healthcare Life Science, USA)] at room temperature overnight. Fifty microliters of the sample in the rehydration buffer was pipetted out and measured for protein concentration using rehydration buffer as the blank (Bradford, 1976). For SDS-PAGE, 10 ml of resolving gel solution [2.5 ml of 1M Tris-HCl (pH8.8), 100 µl of 10% SDS (w/v) (Bio-Rad Laboratories, USA), 3 ml of 40% acrylamide (w/v) (Bio-Rad Laboratories, USA), 50 µl of 10% (w/v) ammonium persulfate (APS) and 5µl tetramethylethylenediamine (TEMED)] and 10 ml of stacking gel solution [2.5 ml of 1M Tris-HCl (pH 6.8), 100 µl of 10% SDS (w/v), 1.3 ml of 40%

acrylamide, 50 µl of 10% APS (w/v) and 10µl tetramethylethylenediamine (TEMED)] (APS and TEMED were not added until gels were ready to pour) were prepared. The protein sample solution was mixed with 2xSDS-PAGE sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol (v/v), 4% SDS (w/v) and 0.1% bromphenol blue (w/v)] at a ratio of 1:1, and heated for 5 min at 95^{o}C in a water bath. After the 12% gel was done, 20 µg of marker (Sigma M3913) or protein sample solution was carefully pipetted into gel sample well. Proteins were first concentrated at a constant voltage of 60V for around 30 minutes at room temperature, and then separated at a constant voltage of 110V for 90 minutes at room temperature. Gels were stained with 0.1% Coomassie Blue R-250 (40% methanol and 10% acetic acid) for 6 hours on an agitator and were de-stained with a solution of 15% (v/v) methanol and 10% (v/v) acetic acid for up to 10 hours. After de-staining, gels were washed three times with distilled water, and then the gel profiles were visualized and scanned with Magicscan (version 4.2) ((UMAX , Techville, Inc., TX, USA).

Software Analysis

Analysis of one-dimensional SDS-PAGE gel images [Figs 3.1 and 3.2] was performed with Phoretix 1D 11.1 software (TotalLab Ltd, New England). Grey 8-bit tiff 1-D gel images were imported to the software. Lanes were detected automatically, and detected lanes were manually verified. Protein band detection was performed with detection parameters set at 100 for minimum slope and 2 for noise reduction. Using these settings, false positive-detected bands were minimized, and the detected protein bands represented bands that can be seen visually. Detection parameters were optimized to visualize each protein band with the software. Detected protein bands were matched to synthetic reference lanes to estimate protein molecular weights.

RESULTS

A few visually qualitative differences of protein expression were identified between *S. alterniflora* and *S. pectinata* (Figs 3.1-3.2). In comparisons of total and heat-stable fraction between fully hydrated *S. alterniflora* and *S. pectinata*, protein bands of 36 kDa, 29 kDa, 24 kDa and 20 kDa were present (Fig. 3.1). For the total fraction, the patterns of protein bands of molecular weight (>65 kDa) look similar (Fig. 3.1). Protein bands of 63 kDa and 60 kDa are dominant in *S. pectinata*, while protein bands of 61 kDa and 58 kDa are dominant in *S. alterniflora*. For the heat-stable fraction, 36 kDa, 29 kDa and 24 kDa bands are present in both species, although 29 kDa and 24 kDa bands are weaker in *S. pectinata*. As in the total fraction, 63 kDa and 60 kDa are more expressed in *S. pectinata*, while the 61 kDa and 58 kDa proteins are more expressed in *S. alterniflora* (Fig. 3.1). In a comparison between dry *S. pectinata* and dry *S. alterniflora* total fractions, 36 kDa, 29 kDa and 24 kDa bands were present in both species. Protein bands of 63 kDa and 60 kDa are present *in S. pectinata*, but 61 kDa and 58 kDa bands are present in *S. alterniflora* (Fig. 3.1). For heat-stable fractions, 63 kDa, 60 kDa, and 36 kDa proteins were weakly present in dry *S. alterniflora* but not clear in dry *S. pectinata*. Protein bands of 29 kDa and 24 kDa were dominant in dry *S. pectinata* but weakly present in dry *S. alterniflora*. In Fig. 3.2, the protein patterns between whole seed and embryo of *S. alterniflora* look similar in both total and heat-stable fractions, except that the 63 kDa, 60kDa and 36 kDa protein bands are more intense in heat-stable fraction of *S. alterniflora* embryo than the whole seed.

Computational analysis of the SDS-PAGE gels (Figs. 3.1 and 3.2) was performed with Phoretix 1D to identify any differential proteome differences between different

physiological comparisons. Figure 3.3 shows the detected protein bands represented with red diamonds and lines of the matched lanes to the reference gel. For the molecular marker [Lane 1], the 1st and 7th diamonds were false positive protein bands. Based on visual judgment, protein bands of 36 kDa, 29 kDa, 24 kDa and 20 kDa were present in all lanes in Figure 3.1. In Figure 3.3, in a comparison of both total and heat-stable fractions of *S. alterniflor*a and *S. pectinata*, a 36 kDa protein band was detected by the software (except the heat-stable fraction of flash dried, non-dormant *S. alterniflora*), which is consistent with visual comparison; however, the band volume is almost twice as large in *S. alterniflora* than *S. pectinata* (Table 3.1). A protein band of ~29 kDa is expressed in all lanes, but there is another band (~27 kDa) shown in the total fraction of fully hydrated *S. alterniflora*. For 20 kDa band, there is no detected band in heat-stable fractions of flash dried *S. alterniflora* and dry *S. pectinata*. In the range between 50 kDa and 60 kDa, there are two major bands (*ca.* 59 kDa and 52 kDa) detected in *S. alterniflora* but three bands (*ca.* 58 kDa, 53 kDa and 49 kDa) in total fractions of fully hydrated and dry, unimbibed *S. pectinata*. However, on lanes of heat-stable fractions of fully hydrated and dry, unimbibed *S. pectinata*, only two (58 kDa and 53 kDa) and one bands (58 kDa) were shown, respectively.

DISCUSSION

In each profile, bands were fairly well- separated and resolved. Proteins migration was even, and no visual small changes in electrophoretic mobility of standard proteins (markers) have been observed, so that constituents of the gel quality from batch to batch are consistent. Noisy backgrounds usually caused by non-proteinaceous material, such as nucleic acids (Görg *et al.*, 2004), were not found in the profiles. Protein bands were

clearly stained, with no uneven staining within individual gels. Distortion of bands, such as band smearing or streaking, was not observed, which means that proteins have been well dissolved in the SDS loading buffer, and few bubbles were formed in the gel.

In order to examine the reproducibility of the gel profiles and software performance, total and heat-stable fractions of fully hydrated and non-dormant *S. alterniflora* were compared between Fig. 3.3 [Lanes 2&3] and Fig. 3.4 [Lanes 2&3]. For the total fraction, there are two bands of 82 kDa and 36 kDa in Fig. 3.3 and one in Fig. 3.4, but the two detected protein bands by the software in Fig. 3.3 are so close that it is difficult to tell whether there are truly two bands. The software analysis of the two total fraction profiles detected 19 protein bands in each gel, but the migration patterns differ somewhat, illustrating the reproducibility challenges involved in SDS-PAGE. For the heat-stable fraction, protein bands of 33 kDa, 27 kDa and 21 kDa were only expressed in Fig. 3.4, but only one protein band was shown in Fig. 3.3. The software analysis scored the presence of 10 bands in one gel [Table 3.1], but 12 bands in the other [Table 3.2]. Overall, while reproducibility of the gel profiles and software performance was satisfactory, it would be problematic to make accurate comparisons between samples using one-dimensional gels.

Figure 3.1 indicates that not many distinctly clear, visually qualitative differences of protein expression pattern were identified between *S. alterniflora* and *S. pectinata* as described above. Comparisons between hydrated *S. alterniflora* and hydrated *S. pectinata*, or between flash dried *S. alterniflora* and 2nd dry down *S. pectinata*, may not be physiologically comparable. *S. pectinata* has been dried down once on the mother plant and then rehydrated again after harvest, while *S. alterniflora* does not undergo maturation

drying on the mother plant. In *S. alterniflora*, drying to moisture contents <40% occurs after harvest. Therefore, use of freshly harvested *S. alterniflora* before submerged storage and *S. spartinae*/*S. pectinata* seeds developing *in planta* would provide the most appropriate physiological comparisons. Alternatively, a comparison between *in planta*-matured and air-dried *S. pectinata/S. spartinae* versus air-dried shatterable ["physiologically mature"] *S. alterniflora* would be physiologically appropriate. Even though the comparison of SDS-PAGE between recalcitrant *S. alterniflora* and orthodox *S. pectinata* indicates they showed differential proteome profiles, it is very difficult to analyze these differentially expressed protein bands with techniques used here, because the proteomes were resolved by one-dimensional electrophoresis and separated based only on molecular weight. It is very likely that one protein band contains many individual proteins. Excision of such bands would present identification problems during subsequent liquid chromatography/mass spectrometry.

By using the software analysis, the effect of drying on the composition of the *S. alterniflora* heat-stable fraction can be estimated by comparison of heat-stable fractions between fully hydrated [Lane 3] and flash dried *S. alterniflora* [Lane 5] [Fig. 3.3, Table 3.1]. The 84 kDa, 71 kDa, 37 kDa, 31 kDa, 25 kDa and 20 kDa bands were present in fully hydrated *S. alterniflora* but missing in flash dried seeds (Table 3.3). On the contrary, 39 kDa, 28 kDa and 24 kDa bands only appear in heat-stable fraction from dry *S. alterniflora* (Table 3.3). Quantitatively, 53-57 kDa and 28-29 kDa proteins have a lower band volume in flash dried seeds, while the volume of 60-64 kDa bands is higher in flash dried *S. alterniflora*.

The effect of drying for the total protein fraction from *S. alterniflora* [Lane 2 vs.

Lane 4]: 88 kDa, 82 kDa, 75 kDa, 40 kDa, 25 kDa and 22 kDa bands were only present in fully hydrated *S. alterniflora*. The band volumes of many proteins increase with drying (Table 3.3).

The effect of heating on proteomic profiles has been examined in both fully hydrated [Lane 2 vs. Lane 3] and flash dried [Lane 4 vs. Lane 5] *S. alterniflora*, and Table 3.3 shows that majority of protein bands disappear or have a lower band volume after heating. It was interesting to note (Table 3.3 summary of Fig 3.3) that either heating or drying stimulated the appearance of 84kD, 39 kDa, and 24 kDa bands among the comparisons. Whether these heat or drying-induced bands represent the same proteins is uncertain.

The majority of total protein fraction appeared or was up-regulated when dry *S. pectinata* seeds were hydrated and stratified to break dormancy [Lane 6 vs. Lane 8] (Table 3.4). Moist chilling increased the number of heat stable *S. pectinata* proteins from four to 13 bands. [Lane 7 vs. Lane 9, Table 3.4]. Among those proteins, the 20 kDa and 31 kDa bands of heat-stable fraction were consistently missing in dried *S. alterniflora* and unimbibed *S. pectinata* but present in fully hydrated seeds (Table 3.3 and Table 3.4). Preparation of the heat-stable fractions from either moist-chilled *S. pectinata* (Tables 3.1 and 3.4; Lane 6 vs Lane 7) or dry, unimbibed *S. pectinata* (Tables 3.1 and 3.4; Lane 8 vs. Lane 9) resulted primarily in the loss or decreased intensity of many protein bands, as would be expected for such a fractionation step.

In a comparison of total fraction between fully hydrated *S. alterniflora* [Lane 2] and *S. pectinata* [Lane 6], 92 kDa, 83 kDa, 57 kDa, 49 kDa, 34 kDa, and 20 kDa bands were present in *S. pectinata* but missing in *S. alterniflora* (Table 3.5), and several protein

bands have a higher band volume in *S. pectinata* than *S. alterniflora*, while protein bands of 88 kDa, 82 kDa, 59 kDa, 33 kDa, 27 kDa and 22 kDa bands were present in *S. alterniflora* but missing in *S. pectinata* (Table 3.5). For the heat-stable fraction of fully hydrated *Spartina* seeds [Lane 3 vs. Lane 7], 75 kDa, 58 kDa, 49 kDa, 40 kDa, and 34 kDa bands were present in *S. pectinata* but not in *S. alterniflora* (Table 3.5). In a comparison between flash dried, nondormant *S. alterniflora* and dry, unimbibed, dormant *S. pectinata*, for the heat-stable fraction [Lane 5 vs. Lane 9], 64 kDa, 57 kDa, 39 kDa and 27 kDa were present in *S. alterniflora* but not in *S. pectinata*, while 58 kDa and 36 kDa bands was uniquely expressed in *S. pectinata* (Table 3.5). For the total fraction [Lane 4 vs. Lane 8], 91 kDa, 31 kDa, 26 kDa, 20 kDa and 19 kDa bands were present in *S. alterniflora* but missing in *S. pectinata*, while 88 kDa, 80 kDa, 57 kDa, 49 kDa, 34 kDa, 23 kDa and 20 kDa were present in *S. pectinata* but not in *S. alterniflora*.

The protein patterns between whole seed and embryo of *S. alterniflora* visually look similar (Fig. 3.2). This suggests that embryo proteins qualitatively represent most proteins in the whole seed, which, however, is contrary to the general case that storage proteins in endosperm are the most distinct bands on whole seed gels (Bewley and Black, 1994). Therefore, most proteins may exist in the embryos, or storage proteins from whole seeds may be masked by other major proteins on SDS-PAGE gels in *S. alterniflora* (Fig. 3.2). Gel profiles between stratified *S. alterniflora* whole seeds and embryos isolated from them were also compared with computational software (Fig. 3.4). For the total protein fraction [Lane 2 vs. Lane 6], at *ca.* 76-78 kDa, only 1 protein band was detected in the whole seed of *S. alterniflora*, but two bands were detected in the embryos. Visually, it is difficult to tell how many protein bands existed in the gel image of the whole seed.

At 53 kDa, two protein bands were visible in the whole seed lane, with only 1 band in the embryo; however, at *ca.* 50 kDa, another band was detected in the embryo but missing in the whole seed. There is one band detected at roughly 20 kDa in the whole seed lane but not in the embryo. Overall therefore, most of the protein bands in the total extract were present in both the endosperm and the embryo, since digital imaging detected the loss of only a 36 kDa and 20 kDa proteins when embryos were excised from the whole seed [Tables 3.2 and 3.3]. For the heat-stable fraction [Lane 3 vs. Lane 7] above 66 kDa, there are two bands, 83 kDa and 74 kDa, detected in the embryo lane but not in the whole seed, but these two missing bands in the heat-stable fraction of the whole seed were present in total fractions of both the whole seed and embryo (Table 3.2). The same situation was observed at 42 kDa in the embryo lane. Quantitative comparisons (Table 3.2) between whole seeds and embryos show inconsistent results: some bands have a higher volume in the whole seed, and *vice versa*. The inconsistency could be explained by the possibility that the embryo proteins are 'diluted' by the endosperm proteins in the whole seed extract so that differential quantitative enrichment might be expected. Because of a lack of enough biological replicates, it is inconclusive to tell whether protein bands of the whole seed or embryo have a significantly higher volume.

In a comparison of protein profiles with and without protease inhibitor for *S. alterniflora* extracts, no visual difference was observed (Fig. 3.2). Proteomic profiles of stratified *S. alterniflora* seed extracts with and without protease inhibitors [Lane 2&3 vs. Lane 4&5 in Fig. 3.4] were also computationally compared. For the total seed extract, 19 bands were present ± protease inhibitor; however, the heat stable fraction without the inhibitor contained only 9 proteins compared to the 12 proteins in the heat stable fraction

with protease inhibitor (Table 3.2). Slight calculated variations in molecular weight were observed, but it is not clear if these are due to partial proteolysis or analytical/software errors. However, for the heat-stable fraction [Lane 3 vs. Lane 5], there are multiple protein bands missing in the proteome without protease inhibitors (~79 kDa, 69 kDa, 24 kDa and 20 kDa). Those missing protein bands may be due to a lack of protease inhibitors. The protease inhibitor cocktail (Sigma, P9599) components all have molecular weights of <1 kDa and are not the source of the additional proteins. The components of P9599 are: 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF); N-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutyryl]-L-leucine hydrochloride (bestatin); pepstatin A; E-64; leupeptin; and 1,10-phenanthroline.

Even though the software is able to quantify the band volume, it is difficult and risky to compare the band volume across the lanes and make conclusions because there is one biological replicate presented here for most treatments, and the variance of the band volume is unknown. However, the qualitative differences [presence vs. absence of protein spots] between treatments suggest that both differential seed desiccation tolerance between *Spartina* species and moist chilling-induced breaking of dormancy are reflected by differences or changes at the proteomic level.

No definitively visual differences in SDS-PAGE protein profiles could be correlated with the extent of desiccation tolerance. The results with software analysis (Figs 3.3, 3.4) are generally consistent with conclusions based on visual comparison (Figs 3.1 and 3.2). For the next step, two-dimensional gel electrophoresis-mass spectrometry and liquid chromatography tandem mass spectrometry will be utilized in the following experiments to overcome the limitations of SDS-PAGE. Two-dimensional gel

electrophoresis, which has a much higher capability in resolving proteins, will be used to investigate the differences in individual protein patterns between recalcitrant *S. alterniflora* and orthodox *S. pectinata* seeds. Based upon these data and densitometric analysis (Tables 3.1-3.5), proteomic differences between the heat-stable fractions of the two *Spartina* species would be expected in subsequent two-dimensional gel electrophoresis investigations. The results of these one-dimensional SDS-PAGE gel comparisons will help to verify the two-dimensional gel comparisons in the future, serving as a guideline to double-check whether or not the differential proteomic expression shown in 1-D gels can also be observed in 2-D gels.

SUMMARY

No definitive differences in SDS-PAGE protein profiles were observed to be correlated with the extent of desiccation tolerance; thus, two-dimensional gel electrophoresis-mass spectrometry and liquid chromatography tandem mass spectrometry will be utilized in the following experiments to overcome the limitations of SDS-PAGE.

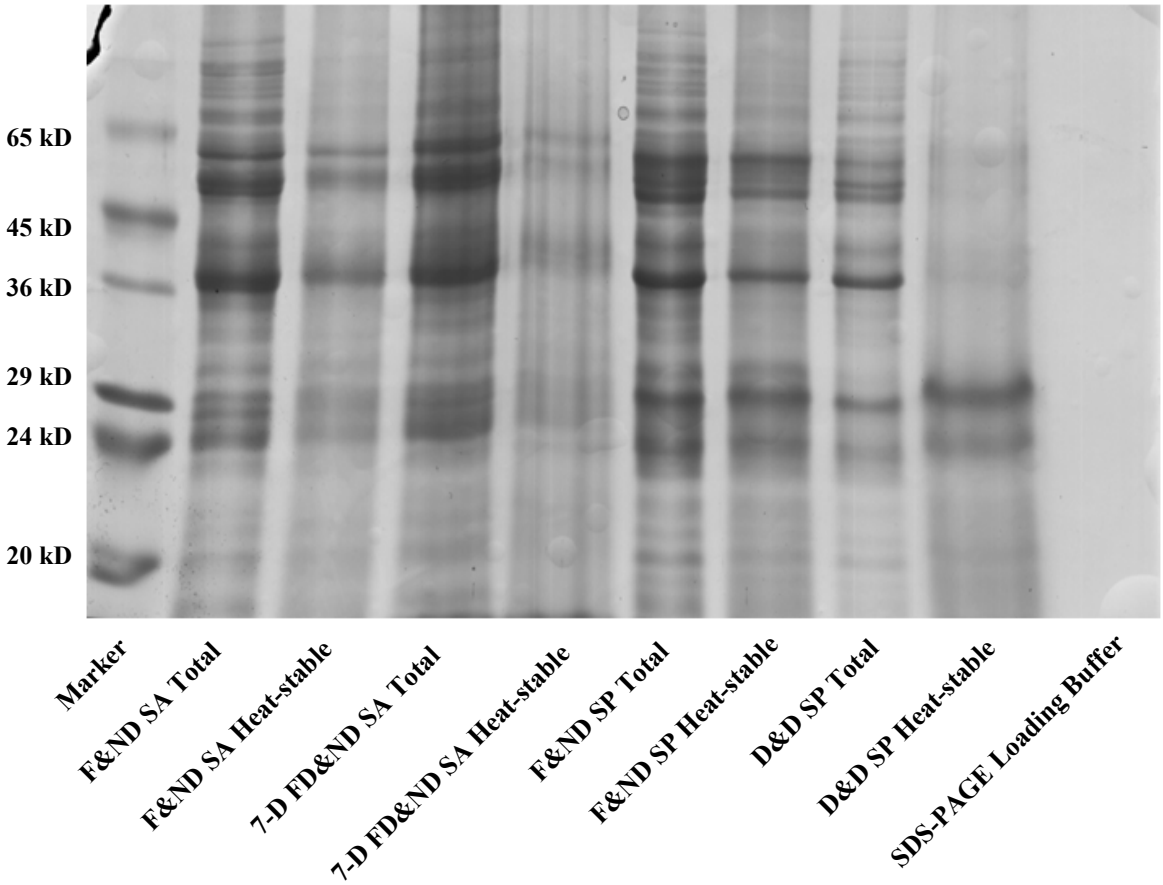


Figure 3.1. SDS-PAGE protein profiles of *Spartin*a seeds (12% polyacrylamide for resolving gel). SA: *Spartina alterniflora*; SP: *Spartina pectinata*; F&ND: fully hydrated and non-dormant; 7-D FD&ND: 7-day flash dried and non-dormant; D&D: dormant and 1st dried. F&ND SA: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2°C [MC=133% (DWB), G=90%, V=90%]. 7-D FD&ND SA: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2°C and flash dried for 7 day at 23°C [MC=13% (DWB), G=5%, V=5%]. F&ND SP: *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), stored dry at -20°C for 17 months, and cold stratified at 2°C for 3 months [MC=119% (DWB), G=80%, V=85%]. D& D SP: *S. pectinata* seeds were purchased from Western Native Seeds, Coaldale, CO (2007), and stored dry at -20°C for 7 months [MC=9% (DWB), G=0%, V=85%].

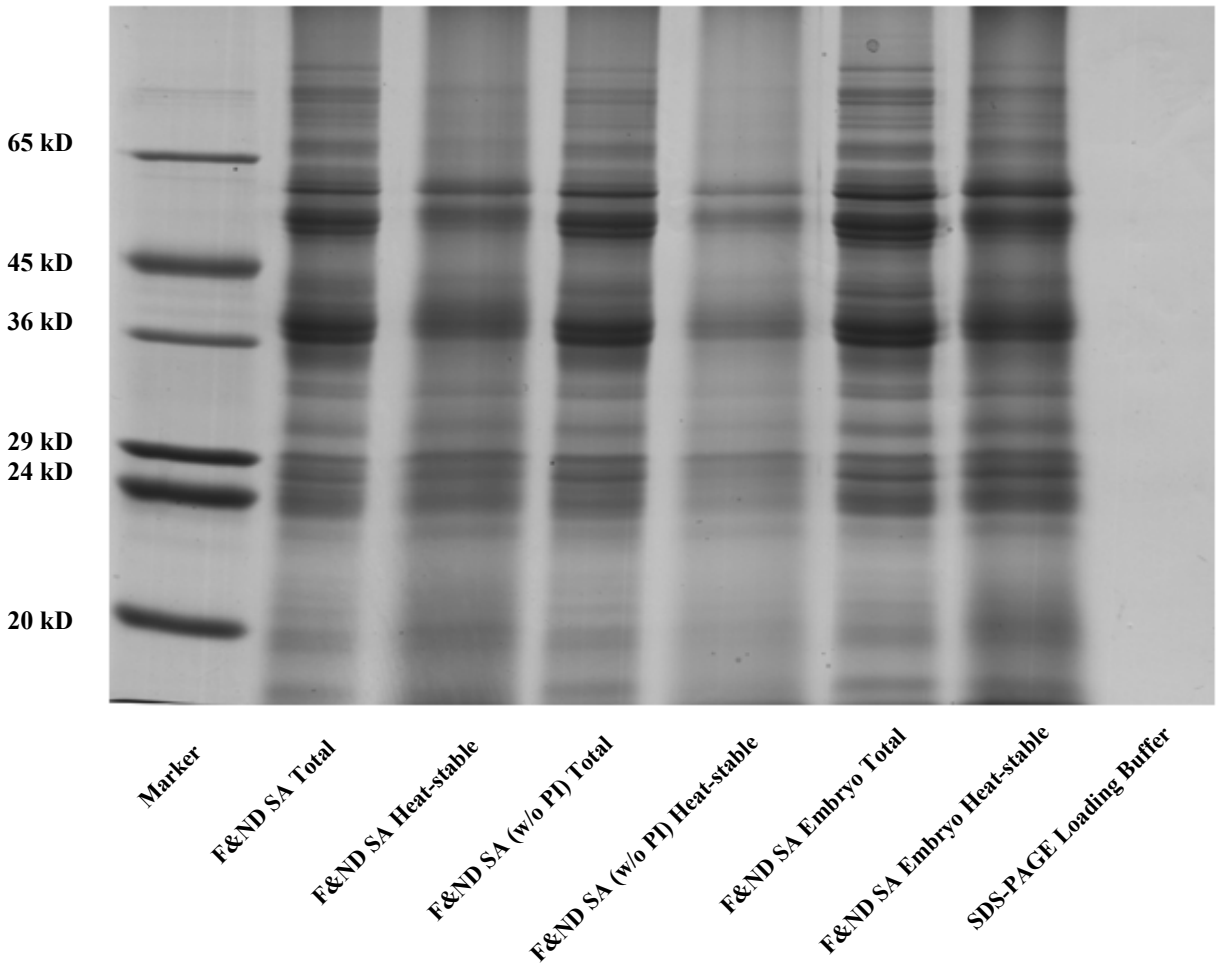


Figure 3.2. SDS-PAGE protein profiles of *Spartina* seeds (12% polyacrylamide for resolving gel). SA: *Spartina alterniflora*; F&ND: fully hydrated and non-dormant; PI: protease inhibitor. F&ND SA: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007) and cold stratified for 6 months at 2°C [MC=133% (DWB), G=90%, V=90%]. When indicated, no protease inhibitor (w/o PI) was added during protein extraction. Embryo: *S. alterniflora* seeds were harvested from Port Fourchon, LA (2007), cold stratified for 1 month at 4°C [MC=133% (DWB), G=90%, V=90%], flash frozen in liquid N_2 and cut to isolate the embryos.

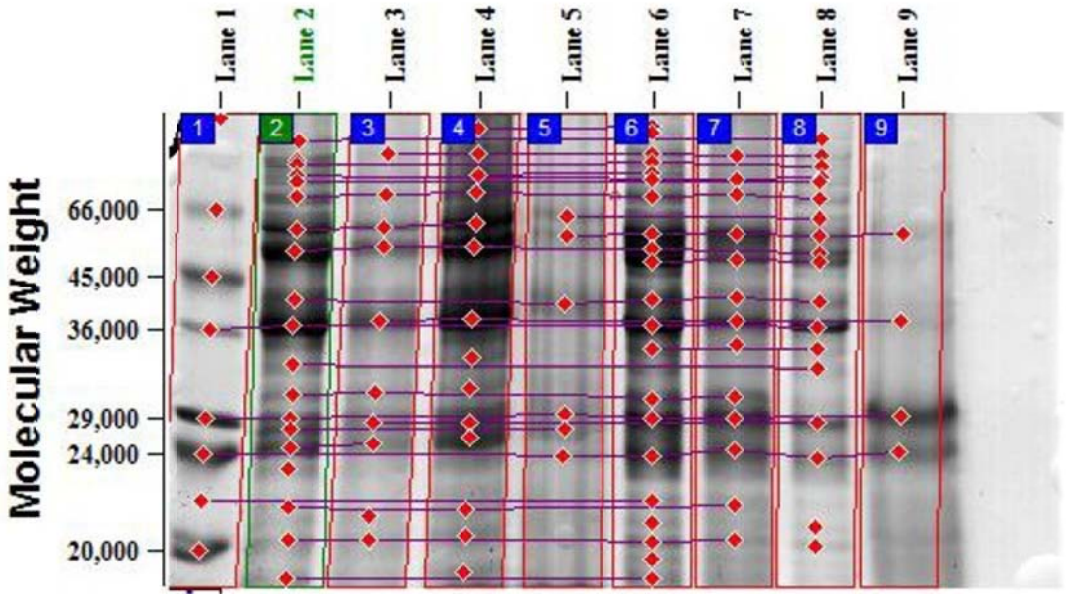


Figure 3.3. Software analysis of the SDS-PAGE gel of Figure 3.1. SDS-PAGE protein profiles of *Spartina* seeds (12% polyacrylamide for resolving gel). SA: *Spartina alterniflora*; SP: *Spartina pectinata*. Lane 1: molecular marker; Lane 2: total fraction of fully hydrated and non-dormant SA (2007) and cold stratified for 6 months at 2^{o}C; Lane 3: heat-stable fraction of fully hydrated and non-dormant SA (2007), and cold stratified for 6 months at 2^{o}C; Lane 4: total fraction of 7-day flash dried & non-dormant SA (2007) and cold stratified for 6 months at 2^{o}C and flash dried for 7 day at 23^{o}C; Lane 5: heat-stable fraction of 7-day flash dried & non-dormant SA (2007) and cold stratified for 6 months at 2^{o}C and flash dried for 7 day at 23^{o}C; Lane 6: total fraction of fully hydrated and non-dormant SP (2007), stored dry at -20^{o}C for 17 months, and cold stratified at 2^{o}C for 3 months; Lane 7: heat-stable fraction of fully hydrated and non-dormant SP (2007), stored dry at -20^{o}C for 17 months, and cold stratified at 2^{o}C for 3 months; Lane 8: total fraction of dry and dormant SP (2007), and stored dry at -20^{o}C for 7 months; Lane 9: heat-stable fraction of dry and dormant SP (2007), and stored dry at -20^{o}C for 7 months.

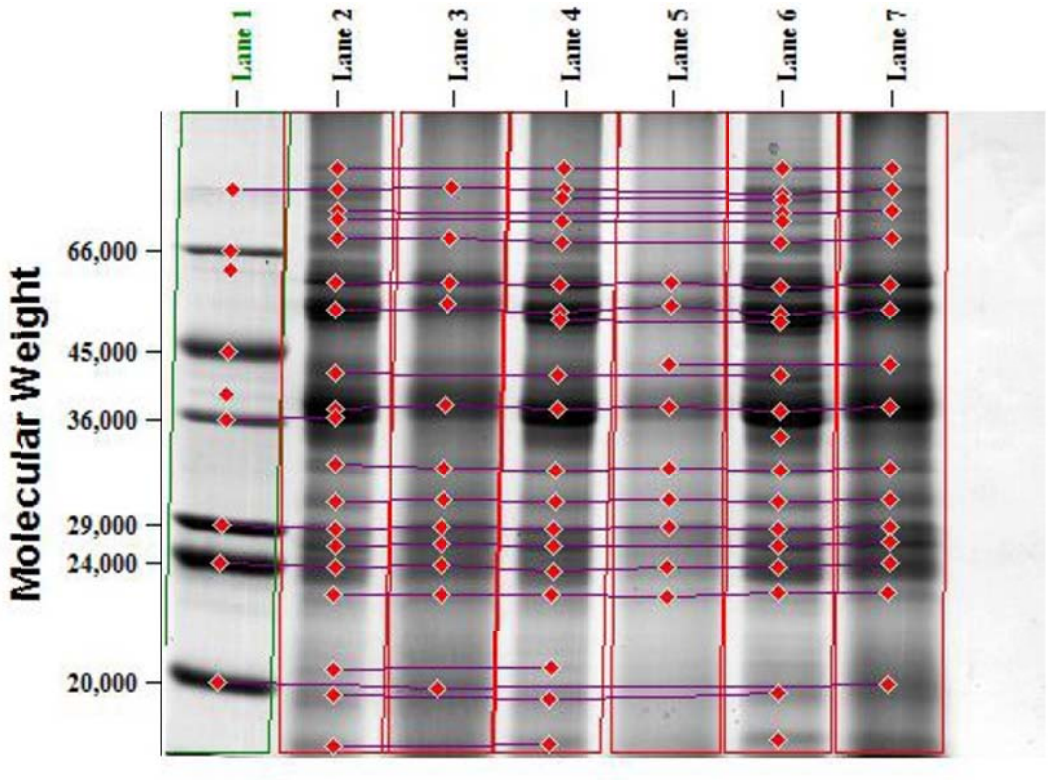


Figure 3.4. Software analysis of the SDS-PAGE gel of Figure 3.2. SDS-PAGE protein profiles of Spartina seeds (12% polyacrylamide for resolving gel). SA: *Spartina alterniflora*. Lane 1: molecular marker; Lane 2: total fraction of fully hydrated and non-dormant SA (2007) and cold stratified for 6 months at 2^{o}C; Lane 3: heat stable-fraction of fully hydrated and non-dormant SA (2007) and cold stratified for 6 months at 2^{o}C; Lane 4: total fraction of fully hydrated and non-dormant SA without protease inhibitor (2007) and cold stratified for 6 months at 2^{o}C; Lane 5: heat-fraction of fully hydrated and non-dormant SA without protease inhibitor (2007) and cold stratified for 6 months at 2^{o}C; Lane 6: total fraction of *S. alterniflora* embryos (2007), cold stratified for 1 month at 4^{o}C, flash frozen in liquid N2 and cut to isolate the embryos; Lane 7: heat-stable fraction of *S. alterniflora* embryos (2007), cold stratified for 1 month at 4^{o}C, flash frozen in liquid N_2 and cut to isolate the embryos.

Table 3.1. Band number, band volume and molecular weight of each gel lane corresponding to Figure 3.3. Band number represents all synthetic protein bands of all lanes; band volume represents the volume of each band that the software calculates; MW represents the putative molecular weight of each protein band that the software calculates based on known molecular markers present (lane 1); - represents no protein band has been detected by the software. SA = *S. alterniflora*, SP = *S. pectinata*.

Band #	**Lane 2**		**Lane 3**		**Lane 4**		**Lane 5**	
	Total fraction fully hydrated & non-dormant SA		**Heat-stable fraction fully hydrated & non-dormant SA**		**Total fraction, non-dormant flash dried SA**		**Heat-stable fraction non-dormant flash dried SA**	
	Band volume	**MW**	**Band volume**	**MW**	**Band volume**	**MW**	**Band volume**	**MW**
1	-	-	-	-	128293	91973.684	-	-
2	-	-	-	-	-	-	-	-
3	143084	88105.263	-	-	-	-	-	-
4	-	-	72605	83684.211	281553	83960.526	-	-
5	123647	82026.316	-	-	-	-	-	-
6	72896	80368.421	-	-	-	-	-	-
7	127661	76776.316	-	-	198324	77328.947	-	-
8	95479	74842.105	-	-	-	-	-	-
9	227736	70144.737	288325	71250.000	328317	71802.632	-	-
10	399783	59580.603	270131	60183.607	502629	61396.880	481573	64152.509
11	-	-	-	-	-	-		
12	664560	52090.231	366188	53467.103	668889	53746.772	318325	57198.978
13	-	-	-	-	-	-	-	-
14	295185	40119.016	-	-	-	-	633973	39390.758
15	815578	36458.614	757858	36957.163	1261598	37168.214	-	-
16	-	-	-	-	-	-	-	-
17	190135	33335.044	-	-	211505	33778.741	-	-
18	253965	31276.270	204090	31482.098	276989	31869.244	-	-
19	194264	29107.861	298879	28315.691	398461	28550.263	544119	29420.191
20	177998	27456.648	-	-	675249	26306.050	241278	27582.235
21	338311	24802.386	261148	25415.859	-	-	-	-
22	188697	22343.358	-	-	-	-	169731	23682.877
23	157536	20298.227	214052	20148.300	384813	20259.332	-	-
24	-	-	-	-	-	-	-	-
25	230360	19990.539	284972	19991.049	276717	19995.096	-	-
26	-	-	-	-	-	-	-	-
27	174889	18867.257	-	-	213927	19150.442	-	-

Table 3.1. (continued from previous page) Band number, band volume and molecular weight of each gel lane corresponding to Figure 3.3. Band number represents all synthetic protein bands of all lanes; band volume represents the volume of each band that the software calculates; MW represents the putative molecular weight of each protein band that the software calculates based on known molecular markers (lane 1); - represents no protein band has been detected by the software. SA = *S. alterniflora*, SP = *S. pectinata*.

Band #	**Lane 6**		**Lane 7**		**Lane 8**		**Lane 9**	
	Total fraction fully hydrated & non-dormant SP		**Heat-stable fraction fully hydrated & non-dormant SP**		**Total fraction dry &dormant SP**		**Heat-stable fraction dry & dormant SP**	
	Band volume	**MW**	**Band volume**	**MW**	**Band volume**	**MW**	**Band volume**	**MW**
1	64051	92802.632	-	-	-	-	-	-
2	72457	90592.105	-	-	87690	88934.211	-	-
3	-	-	-	-	-	-	-	-
4	191357	83960.526	153140	83407.895	145688	83131.579	-	-
5	-	-	-	-	-	-	-	-
6	103890	80921.053	-	-	45839	80092.105	-	-
7	62172	77881.579	-	-	40241	76500.000	-	-
8	98268	75947.368	244047	75671.053	75516	74842.105	-	-
9	238545	70144.737	204028	70973.684	175554	69315.789	-	-
10	-	-	-	-	93225	63230.651	-	-
11	461800	57788.388	492576	58084.927	232561	57198.023	462738	58084.927
12	191823	52635.427	-	-	141687	51818.270	-	-
13	333819	49202.607	212901	49968.671	167407	49202.607	-	-
14	347261	40273.087	221941	40582.888	270614	39678.434	-	-
15	467390	36459.693	493390	36855.883	352673	36271.174	395879	36855.883
16	398048	34364.695	197628	34691.366	104258	34301.749	-	-
17	-	-	-	-	130270	33147.664	-	-
18	248538	30993.199	257163	31067.616	-	-	-	-
19	437405	28778.349	469795	29000	314031	28315.010	746817	29214.139
20	-	-	-	-	-	-	-	-
21	529526	23581.193	567326	24220.837	411414	23286.815	440071	24000
22	-	-	-	-	-	-	-	-
23	202132	20437.311	220478	20320.121	-	-	-	-
24	134677	20056.330	-	-	86240	20019.855	-	-
25	193919	19990.880	189424	19991.051	31489	19990.880	-	-
26	107931	19646.018	-	-	-	-	-	-
27	171004	18831.858	-	-	-	-	-	-

Table 3.2. Band number, band volume and molecular weight of each gel lane corresponding to Figure 3.4. Band number represents all synthetic protein bands of all lanes; band volume represents the volume of each band that the software calculates; MW = putative molecular weight of each protein band that the software calculates based on known molecular markers (lane 1); - represents no protein band has been detected by the software. SA = *S. alterniflora*.

Band #	**Lane 2**		**Lane 3**		**Lane 6**		**Lane 7**	
	Total fraction fully hydrated & non-dormant SA		**Heat-stable fraction fully hydrated & non-dormant SA**		**Total fraction fully hydrated & non-dormant SA Embryos**		**Heat-stable fraction fully hydrated & non-dormant SA Embryos**	
	Band volume	**MW**	**Band volume**	**MW**	**Band volume**	**MW**	**Band volume**	**MW**
1	154979	83242.105	-	-	149535	82800.000	138282	83021.053
2	255839	78600.000	236242	79042.105	181091	77715.789	289742	78600.000
3	-	-	-	-	105480	76389.474	-	-
4	173316	74178.947	-	-	85279	73515.789	233800	73957.895
5	130637	72631.579	-	-	129225	71747.368	-	-
6	312425	68431.579	267802	68652.632	326589	67547.368	320550	68210.526
7	446248	59137.266	449608	59137.266	500425	57975.469	541432	58439.468
8	713349	53213.340	712619	54549.990	370439	52117.901	772494	52993.965
9	-	-	-	-	444418	50187.914	-	-
10	375559	41341.976	-	-	408539	40864.518	229675	42498.721
11	508106	36992.167	1116259	37441.777	797531	36673.187	1204479	37100.158
12	571641	36182.900	-	-	-	-	-	-
13	-	-	-	-	313945	34734.570	-	-
14	242347	33419.355	261128	33229.248	265687	33114.107	290144	33208.451
15	338883	31265.934	294411	31497.775	372613	31341.269	418388	31493.551
16	247549	28459.330	280134	28734.635	227883	28598.310	241562	28734.750
17	217421	26051.359	201569	26515.219	264020	26206.176	316529	26670.228
18	469486	23364.086	408873	23738.019	463125	23363.965	517954	23867.699
19	277842	21056.894	332795	20991.795	334345	21192.364	356616	21123.414
20	265326	19667.129	-	-	-	-	-	-
21	415107	19578.947	965180	19667.129	711357	19614.035	966458	19666.719
22	208282	17894.737	-	-	250544	18140.351	-	-

Table 3.2. (continued). Band number, band volume and molecular weight of each gel lane correspond to Figure 3.4. Band number represents all synthetic protein bands of all lanes; band volume represents the volume of each band that the software calculates; MW = molecular weight of each protein band that software calculates based on known molecular marker (lane 1); - represents no protein band has been detected by the software. SA = *S. alterniflora*.

Band #	**Lane 4**		**Lane 5**	
	Total fraction of fully hydrated & non-dormant SA, without protease inhibitor		**Heat-stable fraction of fully hydrated & non-dormant SA, without protease inhibitor**	
	Band volume	**MW**	**Band volume**	**MW**
1	112263	83021.053	-	-
2	129886	78821.053	-	-
3	108766	76831.579	-	-
4	-	-	-	-
5	138193	72189.474	-	-
6	302295	67768.421	-	-
7	441469	58438.485	333130	58672.021
8	351884	52553.349	519161	53657.762
9	395240	50823.385	-	-
10	356910	41182.018	161090	42498.721
11	1085043	36884.365	709938	37100.158
12	-	-	-	-
13	-	-	-	-
14	232866	33133.260	202070	33208.451
15	333302	31344.907	259679	31493.551
16	194707	28459.330	404038	28734.750
17	239009	26051.359	-	-
18	319632	23012.454	265835	23363.965
19	321522	21056.894	261583	20928.070
20	365780	19667.129	-	-
21	381853	19473.684	-	-
22	232852	17964.912	-	-

Table 3.3. Comparisons of one-dimensional gel profiles of *S. alterniflora* of differential states. Results of software analysis are in Figs 3.3 and 3.4. All detected protein bands were summarized in Tables 3.1 and 3.2. Numbers in the parenthesis represents detected protein band numbers in Tables 3.1 and 3.2.

SDS-PAGE *Spartina alterniflora* comparisons	**Appeared protein bands**	**Disappeared protein bands**	**Increased spot volume**	**Decreased spot volume**	**No change of spot volume**
Figure 3.3	**Molecular weight (kDa)**				
Lane 2 vs. Lane 4 Effect of drying on total fraction profile	92, 84	88, 82, 75, 40, 25, 22	77, 72, 59-61, 37, 29, 26-27, 20 (#23), 19		52-54, 33, 31, 20 (#25)
Lane 3 vs. Lane 5 Effect of drying on heat-stable fraction profile	39, 28, 24	84, 71, 37, 31, 25, 20 (#23), 20 (#25)	60-64	53-57, 28-29	
Lane 2 vs. Lane 3 Effect of heating in fully hydrated *S. alterniflora*	84,	88, 82, 80, 77, 75, 40, 33, 27, 22, 19	70-71,	60, 52-53, 36, 31, 24-25	28-29, 20 (#23), 20 (#25)
Lane 4 vs. Lane 5 Effect of heating in flash dried *S. alterniflora*	39, 24	92, 84, 77, 72, 37, 34, 32	29	54-57, 26-27	61-64
Figure 3.4					
Lane 2 vs. Lane 4 Effect of protease inhibitor on total fraction profile	77, 41	74, 36	37, 21, 20 (#20), 18	83, 79, 53, 28, 20 (#21)	72, 68, 58-59, 33, 31, 23
Lane 3 vs. Lane 5 Effect of protease inhibitor on heat-stable fraction profile	42	79, 69, 27, 20		58-59, 54-55, 37, 33, 31, 24, 21	29
Figure 3.4					
Lane 2 vs. Lane 6 Comparison of total fraction between whole tissue and isolated embryo	76, 50, 35	36, 20	58-59, 41, 37, 31, 26, 21, 19, 18	77-78, 74, 52-53	82-83, 71-72, 68, 33, 28, 23
Lane 3 vs. Lane 7 Comparison of heat-stable fraction between whole tissue and isolated embryo	83, 74, 42		79, 68, 58-59, 53-54, 33, 31, 26, 23,	28,	37, 20-21, 19

Table 3.4. Comparisons of one-dimensional gel profiles of *S. pectinata* of differential states. Results of software analysis are in Fig. 3.3. All detected protein bands were summarized in Table 3.1. Numbers in the parenthesis represents detected protein band numbers in Table 3.1.

SDS-PAGE *Spartina pectinata* comparisons	**Appeared protein bands**	**Disappeared protein bands**	**Increased spot volume**	**Decreased spot volume**	**No change of spot volume**
Figure 3.3	**Molecular weight (kDa)**				
Lane 6 vs. Lane 8 Effect of moist chilling on total fraction profile	92, 30, 20 (#23), 19 (#26), 18	63, 33	83, 80, 76-77, 74-75, 69-70, 57, 51-52, 49, 39-40, 36, 34, 28, 23, 20 (#24), 19 (#25)	88-90	
Lane 7 vs. Lane 9 Effect of moist chilling on heat-stable fraction profile	83, 75, 70, 49, 40, 34, 31, 20, 19		36, 24	29	58
Lane 6 vs. Lane 7 Effect of heating in fully hydrated *S. pectinata*		92, 90, 80, 77, 52, 20 (#24), 19 (#26), 18	75, 23-24	83, 70, 49, 40, 34	57-58, 36, 30-31, 28-29, 20 (#23), 19 (#25)
Lane 8 vs. Lane 9 Effect of heating in dry *S. pectinata*		88, 83, 80, 76, 74, 69, 63, 51, 49, 39, 34, 33, 20, 19	57-58, 36, 28-29		24

Table 3.5. Comparisons of one-dimensional gel profiles between *S. pectinata* and *S. alterniflora* of differential states. Results of software analysis are in Fig 3.3. All detected protein bands are summarized in Tables 3.1 and 3.2. Numbers in the parenthesis represents detected protein band number in Table 3.1. SA = *S. alterniflora*.

SDS-PAGE *S. pectinata* vs. *S. alterniflora* comparisons	**protein bands present in SA**	**protein bands absence in SA**	**Increased spot volume in SA**	**Decreased spot volume in SA**	**No change of spot volume**
Figure 3.3	**Molecular weight (kDa)**				
Lane 6 vs. Lane 2 Total fraction of fully hydrated *S. pectinata* & *S. alterniflora*	88, 82, 59, 33, 27, 22	92, 90, 83, 57, 49, 34, 20 (#24), 19 (#26)	76, 52, 36, 19 (#25)	80, 40, 28-29, 23-24, 20 (#23)	74-75, 70, 30-31, 18
Lane 7 vs. Lane 3 Heat-stable fraction of fully hydrated *S. pectinata* & *S. alterniflora*	60, 53	75, 58, 49, 40, 34	70-71, 36, 19	83, 31, 28-29, 24-25	20
Lane 8 vs. Lane 4 Total fraction of dry *S. pectinata* & flash dried *S. alterniflora*	91,31, 26, 20 (#23), 19 (#27)	88, 80, 57, 49, 39, 34, 23, 20 (#24)	83, 76-77, 69-71, 61-63, 51-53, 36-37, 33, 28, 19 (#25)		
Lane 9 vs. Lane 5 Heat-stable fraction of dry *S. pectinata* & flash dried *S. alterniflora*	64, 57, 39, 27, 23	58, 36		29	

CHAPTER 4
CONCLUSIONS

An extraction protocol for water soluble and heat-stable *Spartina* seed proteins was developed from a review of the literature and experimentation. Processing parameters required to obtain a consistent heat stable fraction were specifically optimized: heating temperature and duration, duration of low temperature precipitation of denatured proteins after heating, the centrifugation regime needed to precipitate denatured proteins, as well as the need for and amount of protease inhibitor. As a result, the following protocol will be used in future *Spartina* seed research. Seed tissues will be ground in liquid nitrogen, transferred to a glass homogenizer and homogenized with 3 ml of ice cold HEPES buffer (pH 7.5) and 50 µl of protease inhibitor (stored at -20^{o}C) added. The protein extract will then be transferred to a clean plastic tube (15ml), and the homogenizer washed with extra 2 ml of HEPES buffer (pH 7.5). The protein extract will be centrifuged at 14,000g, at 4°C for 20 min, twice. The protein supernatant after centrifugation is heated at 95^{o}C for 40 min and ice-incubated for 30 min. Then, heat denatured proteins are spun down and removed by centrifuging at 20,000 g for 40 min.

Optimization of extraction parameters provided protein content data, which aided the selection of the most comparable seed treatments among the recalcitrant and orthodox species for further proteomics work. Protein yields differed among *Spartina* seeds/embryos of various physiological states. The percentage of heat-stable protein fractions is higher in fully hydrated, dormant and non-dormant *S. pectinata* than *S. alterniflora*, but dry and dormant *S. pectinata* has a lower percent heat-stable fraction than *S. alterniflora*. For *S. alterniflora*, drying following extended hydration reduces the amounts of total and heat stable proteins; the transition from the dormant to non-dormant

state also reduces protein yields. Therefore, one must carefully select the storage conditions for seeds to fairly compare the seed proteomes of various *Spartina* species to increase the chance that the identified protein differences are associated with desiccation tolerance rather than dormancy status or seed vigor. Based upon the trends of the analyses reported here, mature harvest-dried, *S. pectinata* and *S. spartinae* seeds will be used and compared with mature harvest-dried *S. alterniflora* seeds to identify proteins associated with desiccation tolerance. If the *S. alterniflora* seeds were to be artificially maintained in a fully hydrated state at 2^{o}C to maintain recalcitrant seed viability, protein differences might occur due to the transition from the dormant to the non-dormant state, as well as further progression towards germination. If S. *pectinata* seeds were also stored hydrated at 2^{o}C, similar transitions might occur, and there is no reliable basis to assure that they could be sampled at a physiological stage comparable to *S. alterniflora*. Furthermore, if *S. pectinata* seeds were fully rehydrated and then dried, this would represent a second cycle of dehydration, which would have no equivalence in the processing of recalcitrant *S. alterniflora* seeds.

Analysis of proteins by SDS-PAGE provided reproducible profiles, but no dramatic qualitative differences. In the future, two-dimensional gel electrophoresis-mass spectrometry and liquid chromatography tandem mass spectrometry will be utilized to overcome the limitations of SDS-PAGE. Two-dimensional gel electrophoresis, which has a much higher capability in resolving proteins, will be used to investigate the differences in individual protein patterns between recalcitrant *S. alterniflora* and orthodox *S. pectinata* seeds.

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APPENDIX A
SUPPORTING FIGURES AND TABLES

Table A-1. Protein concentrations of total fractions and heat-stable fractions (95^0C and 100^0C for 40 minutes) for fully hydrated and dormant *S. alterniflora*. *S. alterniflora* seeds were harvested from Port Fourchon, LA (2009) and cold stratified for 1 week at 2^0C [MC=124% (DWB), G=0%, V=100%]. Each mean represents 3 analytical replicates. Error bars ± SE. Notebook 7: 14.

Samples	Protein concentration (µg/seed)
total soluble protein	53.9 ± 0.7
after 95°C, 40 min	31.1 ± 0.5
after 100°C, 40 min	30.9 ± 0.8

Table A-2. An example of a sample calculation for taking Bradford assay data and converting it to μg/seed. Notebook 5: 35-36.

1), standard curve drawn by SigmaPlot (version 11.0): Y = 0.7564X + 0.07038; Y: absorbance; X: protein concentration
2), $Absorbance_{595}$ of protein sample: Y = 0.529
3), protein concentration calculated from standard curve: X = (0.529-0.07038) ÷ 0.7564, X = 0.6063 (mg/ml)
4), volume of extraction buffer: 4.5 ml
5), total protein amounts in the extraction buffer: 4.5 (ml) x 0.6063 (mg/ml) = 2.7284 (mg)
6), number of *S. alterniflora* seeds used for protein extraction: 50
7), protein amount per seed: 2.7284 (mg) ÷ 50 = 0.05457 (mg/seed) = 54.57 (μg/seed)

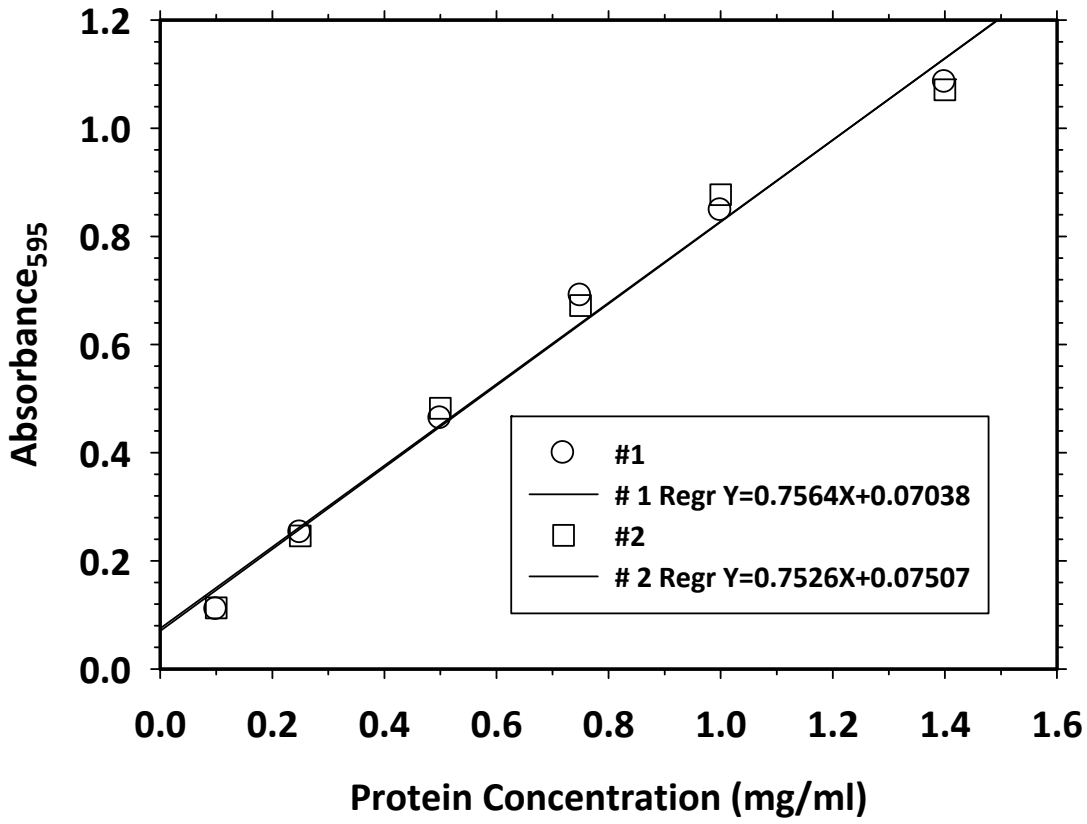

Figure A-1. Standard curves of BSA dissolved in HEPES buffer (pH 7.5). #1: standard curve of bottle #1 of Bradford reagent purchased from Sigma-Aldrich on Jan 5th 2009; #2: standard curve of bottle #2 of Bradford reagent purchased from Sigma-Aldrich on Jan 5th 2009. Absorbance was taken at 595 nm after a 5 minute reaction at 23^{0}C. Notebook 5: 35-37.

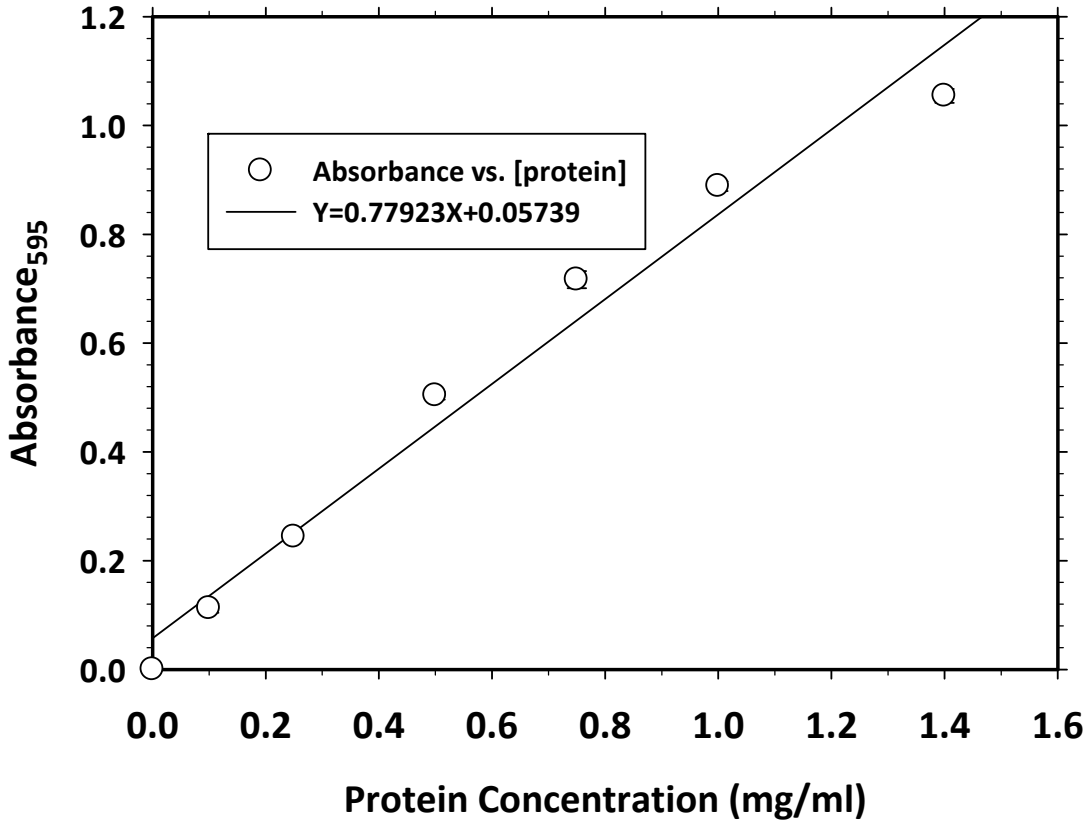

Figure A-2. Standard curves of BSA dissolved in rehydration buffer (pH 8.5). Absorbance was taken at 595 nm after a 5 minute reaction at 23^0C. Notebook 4: 7.

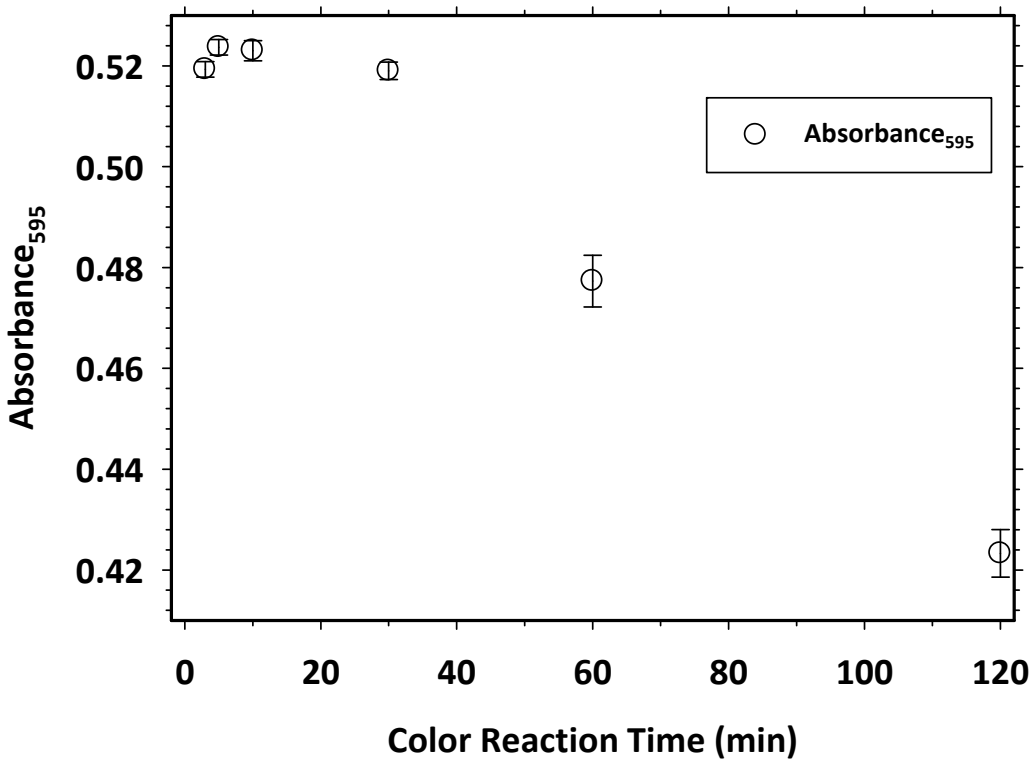

Figure A-3. Optimization of Bradford reagent colorimetric reaction time, using absorbance of protein sample from fully hydrated *S. alterniflora* (Port Fourchon, Louisiana, 2007). Each mean represents 3 analytical replicates. Absorbance was taken at 595 nm at 23^0C. Error bars ± SE. Notebook 2: 5.

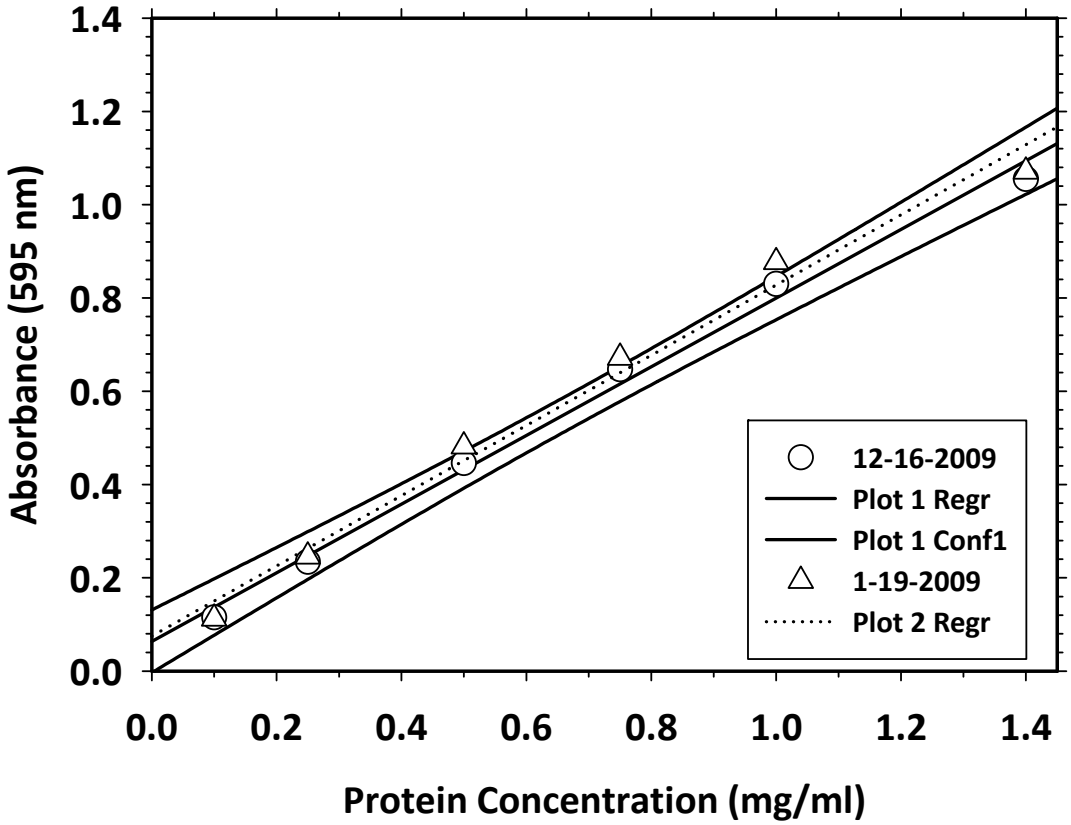

Figure A-4. The effect of storage of the Bradford reagent on the detection sensitivity. Bradford reagent was purchased on Jan 5th, 2009 and stored at 4^{0}C. The 1st standard curve experiment was finished on Jan 19th, 2009 and the 2nd standard curve experiment was finished on Dec 16th, 2009. The 95% confident intervals of the 1st standard curve are shown. Absorbance was taken at 595 nm after a 5 minute reaction at 23^{0}C. Notebook 7: 21-22.

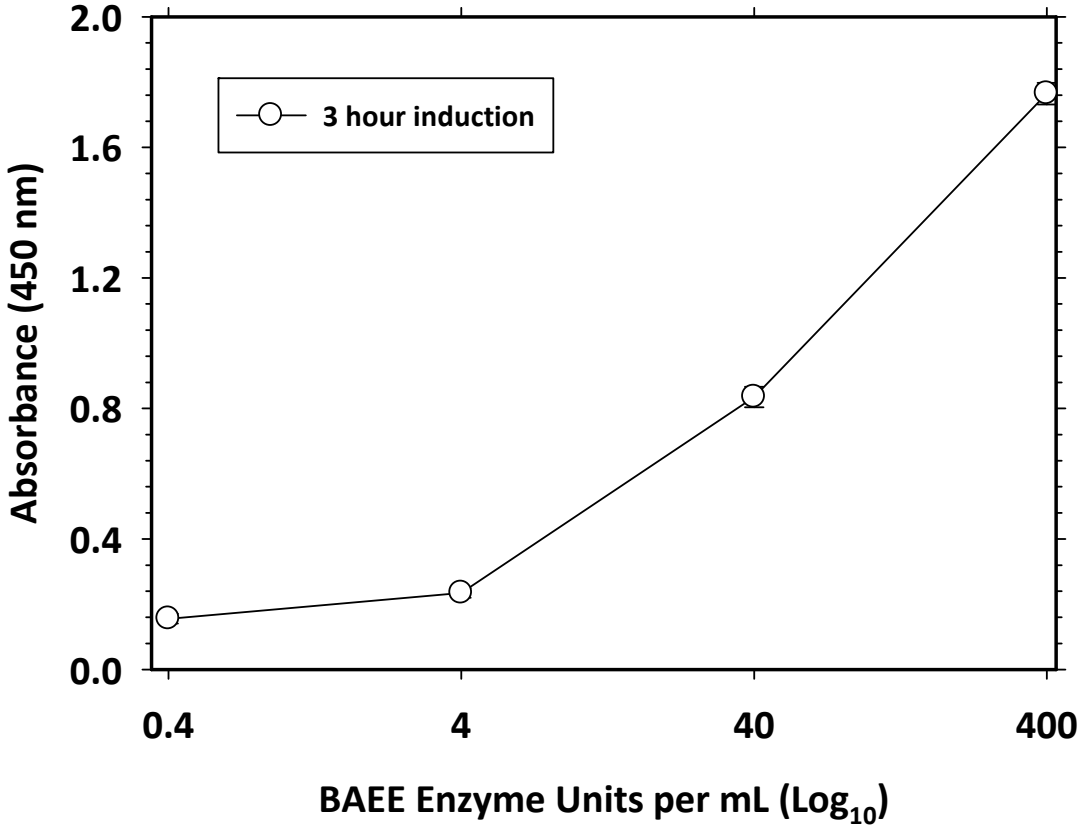

Figure A-5. Standard curve of the PDQ^{TM} colorimetric assay. Trypsin supplied as control (0.7 mg/ml, 1420 BAEE/mg) was diluted to a ten-fold series (0.4 to 400 BAEE/ml, 0.28 to 280 mg/ml). Tris buffer alone is used as blank control. Each mean represents 3 analytical replicates. Absorbance was taken at 450 nm after a 3-hour reaction at 37^0C. Error bars ± SE.

APPENDIX B
LABORATORY NOTEBOOK LOCATIONS FOR DATA IN FIGURES AND TABLES IN THE BODY OF THE THESIS

1. Data corresponding to figure 2.2. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations in the fully-hydrated and non-dormant *S. alterniflora* seeds. Notebook 3: 23, 48, 49.
2. Data corresponding to figure 2.3. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for fully-hydrated and dormant *S. alterniflora* seeds. Notebook 3: 27, 53, 54.
3. Data corresponding to figure 2.4. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for 7-day-flash dried and non-dormant *S. alterniflora* seeds. Notebook 3: 89-91.
4. Data corresponding to figure 2.5. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for 7-day-flash dried and dormant *S. alterniflora* seeds. Notebook 6: 91.
5. Data corresponding to figure 2.6. Protein concentrations of total and heat-stable fractions at different heating temperatures for different durations for dry and aged *S. alterniflora* seeds. Notebook 3: 21, 52.
6. Data corresponding to figure 2.7. Protein concentrations of total and heat-stable fractions at 95^0C for different durations for fully-hydrated and dormant/non-dormant *S. alterniflora* isolated embryos. Notebook 6: 93-94.
7. Data corresponding to figure 2.8. Protein concentrations of total fractions and heat-stable fractions that were at different heating temperatures for different durations for fully-hydrated and non-dormant *S. pectinata* seeds. Notebook 3, 20,

57, 58.

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VITA

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