IDENTIFICATION OF PROTEINS RELATED TO ACCELERATED AGING IN SUNFLOWER VIA MALDI-TOF

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Abstract

Accelerated aging of sunflower (*Helianthus annuus* L.) seeds resulted in a significant decrease of germination rates and total protein contents. The correlation index between the germination rates and the protein contents was 0.937. Two-dimensional electrophoresis was employed to separate proteins of embryonic axes of aged seeds. The results indicated that the abundance of some proteins when increased from 2d to 4d accelerated aging. Two proteins were identified by using MALDI-TOF in this study. One protein matched 26.2% peptide with resistance gene analog NBS4, and the other protein shared 23.5% peptide with resistance gene analog NBS5. These two proteins could be used to alleviate the damage caused by accelerated aging.

Introduction

The quality of seed is important for the seed industry and for farmers. The process of seed aging may bring deterioration and decreased germination ability of seeds (Walters, 1998). The deleterious effects are associated with the damage occurring not only at the membrane and nucleic acid levels, but also at the protein levels (Gidrol et al., 1989; Fujikura et al., 1995; Kalpana and Rao, 1997; Guy and Black, 1998; Benamar et al., 2003). Hsu et al. (2003) found that accelerated aging in bitter gourd seeds (*Momordica charantia* L.) resulted in decreased activities of several free radical and peroxide-scavenging enzymes. The contents of storage proteins in cotyledons of peanut seeds (*Arachis hypogoea* L.) were observed to decline followed by a vigor loss in seed growth potential. Moreover, many proteins, such as HSPs and LEAs, were reported to alleviate damage made by accelerated aging (Houde et al., 1992).

Sunflower is an important oil crop in the world. The seed of this species is relatively short-lived and susceptible to seed aging. Little information about protein synthesis and its functions involved in aging seeds of sunflower is available (Ellis et al., 1995; Peter et al., 1998; Hsu et al., 2003). A new methodology, matrix-associated laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy, may provide a powerful tool for the analysis of proteome changes in aging seeds. In this paper we investigate the variation in protein synthesis and production during accelerated aging by using the MALDI-TOF in order to collect more data on longevity of sunflower seeds.

Materials and Methods

Sunflower Seed Aging. Sunflower (*H. annuus* L.) seeds of the genotype S12, harvested in 2003, were provided by our lab. Accelerated aging was performed by incubating sunflower seeds at 42C and 100% RH for 8 days. After aging, the seeds were dried at 30C to their original weights. Aged and unaged seeds were sealed in aluminum foil bags coated with polyethylene, and stored at 5C for use within a month following aging.

Germination Tests. Germination tests were performed in triplicate on 50 sunflower seeds without seed coats. Seeds were incubated in a chamber with a 12 h photoperiod (about 225-250W/m sq. light intensity) at 20C. Seeds were counted daily for germination for 7 days. To characterize cumulative germination curves, we registered the number of emergences every day, and calculated the average of three replicates. Mean emergence time (MET) was calculated using the formula of Ellis and Roberts (1980). Data were analyzed with SPSS 10.0 for Windows (Release 10.01, Chicago, Illinois, USA).

Protein Extraction. After 8 h imbibition at 20C in aseptic water, seeds were chosen randomly for protein extraction. Embryonic axes were excised from unaged and aged seeds. Unaged, 1d, 3d, and 5d aged embryonic axes were ground in liquid nitrogen to a fine powder. Total proteins were extracted from milled powders at 4C by thiourea/urea lyses buffer (Harder et al., 1999). The mixture was stirred for 20 min and centrifuged (40,000×g) at 4C for 20 min. The final supernatant was collected and stored at -20C for use. Protein concentrations were measured by the method of Bradford (1976).

Two-Dimensional Electrophoresis and Image Analysis. Two-dimensional electrophoresis and the staining method were performed according to the method of Simpson (2003). Image analysis was performed with Melanie 3.0 software (GeneBio, Geneva), according to the instructions. After spot detection and background subtraction, gels were aligned and matched, and the quantitative determination of the spot volumes was performed. Specific spots were described as showing variations during accelerated aging when their volumes were significantly different in the three analyzed gels from each extraction. We chose two interesting spots with gradual increase in volume during accelerated aging to identify by MALDI-TOF analysis according to the method described by Pappin (1993).

Results

Changes in Germination Rates, Protein Contents and MET Influenced by Accelerated Aging. Generally, accelerated aging decreased the germination rates (Figure 1). In the first 2 days of accelerated aging, the germination rates decreased quickly. However, the germination rates were reduced slowly in the next 3 days. The germination rates dropped faster in the following 4 days, and only 30.67% of 8 day aged-seeds showed normal emergence. The protein contents of embryonic axes shared a similar trend with germination rates after accelerated aging (data not shown). As shown in Figure 2, mean emergence time (MET) was prolonged from 2.66 days to 3.73 days, suggesting a vigor loss of seeds during accelerated aging, while MET had little change or was stable from 2d to 4d accelerated aging.

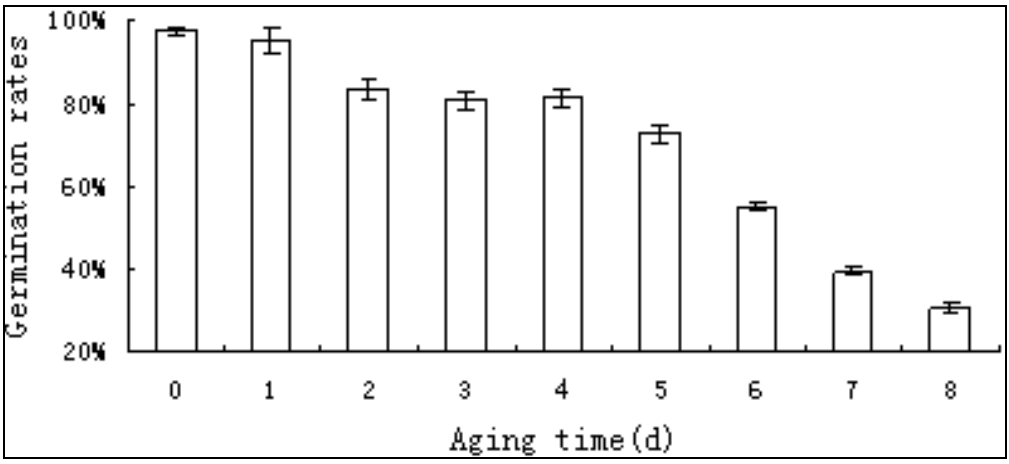

Figure 1. Changes in germination rates during accelerated aging of sunflower seeds.

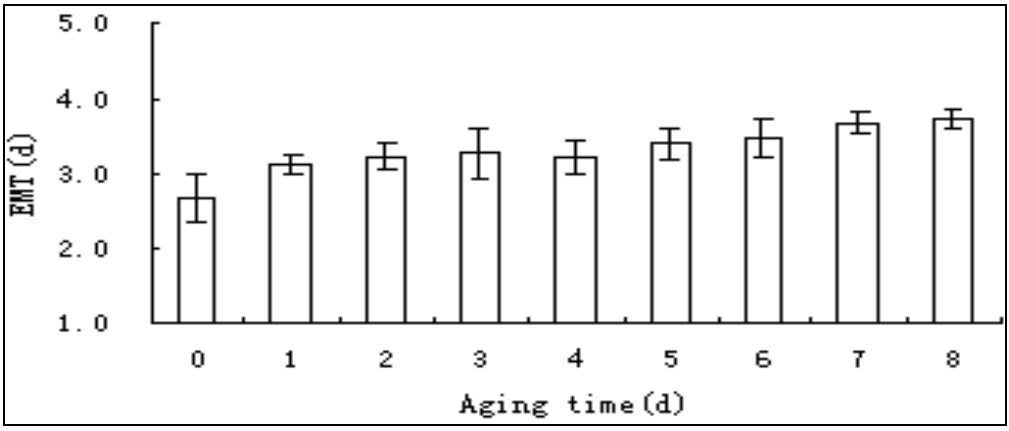

Figure 2. Mean emergence time (MET) of sunflower seeds influenced by accelerated aging.

The correlation index between the germination rates and the protein contents was 0.937, and the two factors were found to be positively and significantly correlated with each other at the 0.01 level. At the same time, the linear relationship between the germination rates and the protein contents was analyzed and the linear function was y= 11.985x+11.727. These results suggested that sunflower seeds lose protein and germination viability simultaneously, and the trends were synchronous.

Identification of Proteins Correlated to Accelerated Aging. Two-dimensional electrophoresis was used to analyze the protein variation in the unaged (A); 1d aged (B); 2d aged(C); and 4d aged (D) sunflower embryonic axes. A total of 369 spots were visible in the proteome of unaged and aged sunflower embryonic axes (Figure 3). Some abundant proteins (MW>43.0kDa and MW<97.0kDa) were marked by ellipses and they varied in the progress of aging. Some proteins' abundance increased, many disappeared and others appeared from 0d to 4d. For example, spot 14 existed in Figure 3A but disappeared in the other gels, which

suggested that spot 14 was sensitive to accelerated aging. In addition, spots 19 and 41 were present in Figures 3C and 3D, respectively and were absent in unaged embryonic axes (Figure 3A). There were 5 proteins (spot 25, 26, 93, 94 and 115) with MW<43.0kDa in the aged embryonic axes whose abundance increased gradually with respect to unaged embryonic axes. Eventually, the abundance of these proteins strongly increased after 5d accelerated aging. Two proteins (spot 25 and spot 26, in Figure 4) were analyzed via matrix-assisted laser desorption-ionization-time-of-flight mass spectrum (MALDI-TOF MS) to obtain their peptide mass fingerprinting (PMF) (Table 1). The results suggested that 5 and 6 peptides in spot 25 and 26 were completely matched with the peptides of resistance gene analogs NBS4 and

Table 1**.** Sunflower embryonic axis polypeptides whose abundance increased during aging.

No.	Experimental molecular weight (kDa)	Experimental p*I*	Protein name	Coverage (%)	Theoretical molecular weight (kDa)	Theoretical p*I*	Accession
25	35.8	8.32	resistance gene analog NBS4	26.2	32.3	7.16	gi|15787895
26	35.2	8.95	resistance gene analog NBS5	23.5	31.2	8.25	gi|15787897

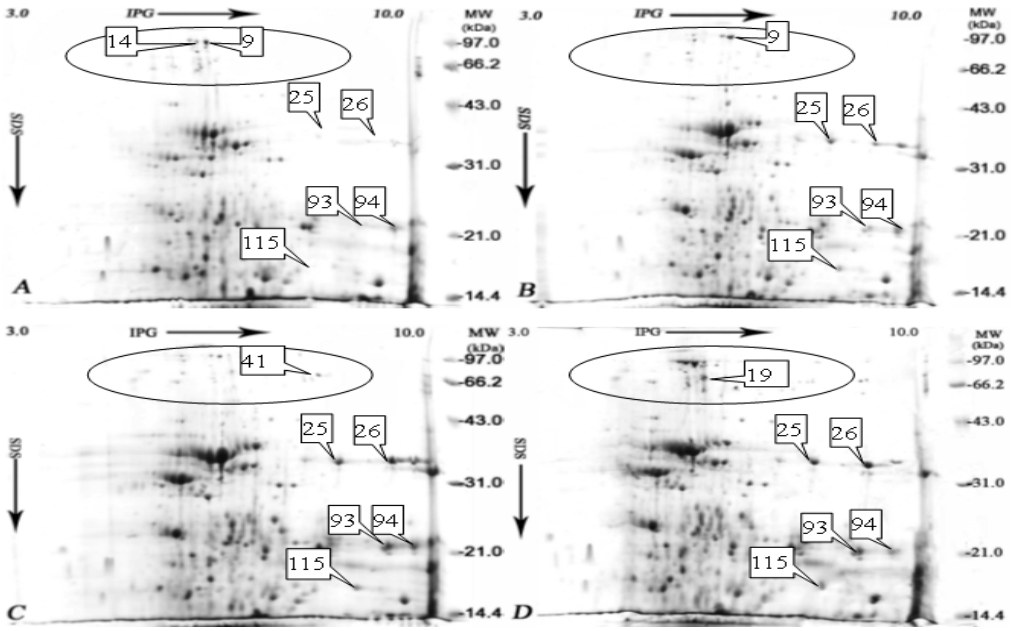

Figure 3. Characterization of unaged (A), 1d-aged (B), 2d-aged(C), 4d-aged (D) sunflower embryonic axis proteins whose abundance increased during accelerated aging.

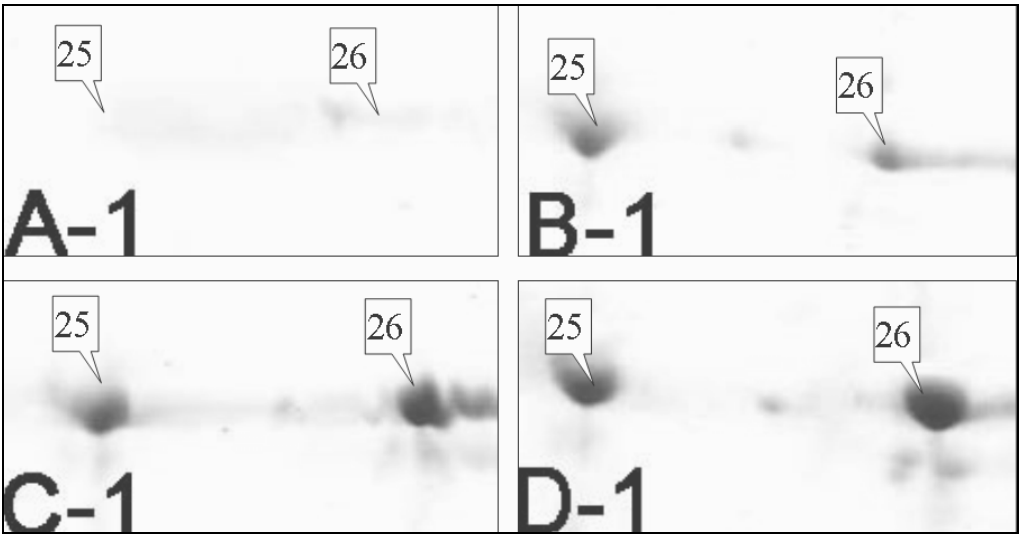

Figure 4. Enlarged windows of new proteins (spot 25 and spot 26) whose abundance increased in unaged (A-1), 1d-aged (B-1), 2d-aged(C-1), and 4d-aged (D-1) sunflower embryonic axes with molecular weights between 31.0kDa to 43.0kDa. The labeled protein (spot 25 and spot 26) was identified by matrix-assisted laser desorption-ionization-time-of-flight (MALDI-TOF) analysis.

NBS5, respectively. In addition, coverage between protein 25 and resistance gene analog NBS4 was 26.2%, and coverage between protein 26 and resistance gene analog NBS5 reached 23.5%.

Discussion

Accelerated aging of seeds induced by several days of exposure to high temperature and high humidity is recognized as an indicator of seed vigor and storability (Bernal-Lugo and Leopold, 1995; Hsu et al., 2003). The seeds under accelerated aging showed a marked depression in their ability to germinate and a reduction of protein content. In this study, the germination rates decreased dramatically, protein content was reduced and MET was prolonged after accelerated aging. This result was similar to that of McDonald (1999). Moreover, our results showed that the correlation between the germination rates and the protein contents of aged sunflower seeds was positively and significantly correlated with each other. Also, it was remarkable that there was a phase in which the germination rates and the protein contents decreased more slowly than in other phases.

Two proteins were identified using the technology of MALDI-TOF in this study. The abundance of the two proteins increased during aging. One protein we chose matched 26.2% peptide with resistance gene analog NBS4, and the other protein shared 23.5% peptide with resistance gene analog NBS5. Nucleotide-binding sites (NBS) belonged to the protein family which was encoded by plant disease resistance genes. With the progress of accelerated aging, it could be possible for these two proteins to protect seed vigor or alleviate the damage caused

by accelerated aging. Obviously, these two proteins could be additional indicators of the degree of seed deterioration in sunflower seeds (Guy and Black, 1998).

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