第 31 卷 第 2 期
2005 年 2 月 177～182 页

作 物 学 报
ACTA AGRONOMICA SINICA

Vol. 31, No. 2
pp. 177 - 182 Feb., 2005

Melon (Cucumis melo L.) Cultivar Identification by Reversed-phase High Performance Liquid Chromatography of Seed Proteins

WANG Qiang[1], RUAN Xiao[1], CHEN He[2], SUN Xiao-Lu[2], SHENG Jian-Dong[3], YAN Qi-Chuan[1]

([1] *Department of Biological and Pharmaceutical Engineering, Ningbo Institute of Technology, Zhejiang University, Ningbo* 315100, *Zhejiang*; [2] *Testing Center, Xinjiang Academy of Agricultural Science, Urumqi* 830050, *Xinjiang*; [3] *Department of Environment, Xinjiang Agriculture University, Urumqi* 830052, *Xinjiang, China*)

Abstract: Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) of cereal grain proteins has been receiving much attention in the agricultural literature published in recent years. A study of melon (*Cucumis melo* L.) hybrid parents S ♂ and S ♀ and F_1 hybrids of K96-8 ×6-1, T-5 ×red1-28 and 94 ×8-1 was undertaken to determine if RP-HPLC could be adapted to the cultivar identification of melons. Chromatograms of the three inbred lines and F_1 hybrids of cultivars were shown to be different and were used to characterize those cultivars. RP-HPLC was shown to be a quick, replicable and reliable method of melon cultivar identification for general screening of seed lots.

Key words: Melon (*Cucumis melo* L.); Cultivar identification; Reversed-Phase High Performance Liquid Chromatography

甜瓜种子醇溶蛋白的反相 HPLC 分析鉴定

王 强[1] 阮 晓[1] 陈 贺[2] 孙晓路[2] 盛建东[3] 颜启传[1]

([1] 浙江大学宁波理工学院,生物与制药工程系,浙江宁波 315100; [2] 新疆农业科学院分析测试中心,新疆乌鲁木齐 830050; [3] 新疆农业大学,资源环境学院,新疆乌鲁木齐 830052)

摘 要:针对目前国内甜瓜的主栽品种 K96-8 ×6-1、T-5 ×red1-28 和 94 ×8-1 的自交系及杂交种种子醇溶贮藏蛋白进行反相 HPLC 法分离。结果表明,其色谱图明显不同,可以用于该品种(系)的鉴定,认为 RP-HPLC 技术是一种快速、准确、可信的甜瓜品种鉴定手段。

关键词:甜瓜;品种鉴定;反相高效液相色谱

中图分类号:S652, O657.7

Cultivar verification is an important area of study in seed analysis[1]. Alcohol soluble seed storage proteins are genetically stable molecules, which can be analyzed by electrophoresis or HPLC to identify cultivars. Electrophoresis studies greatly enhanced the investigation and understanding of cereal proteins. Brink, Price, Nguyen, Fuerst and Martinez (1989) used isoelectric focusing of zeins and an immunoassay system to evaluate the genetic purity of maize (*Zea mays* L. subsp. *mays*)[2]. New methods such as RP-HPLC and capillary electrophoresis have been applied to identify cultivars. Dinelli G and Bonetti A (1992)[3] used capillary electrophoresis as an identification tool for *Phaseolus vulgaris* L. cultivars. Both the International Seed Testing Association and the Association of Official Seed Analysis are interested in applying new biochemical methods to the analysis of seeds and the standardization of these techniques for cultivars identification.

Reversed-phase high performance liquid chromatography (RP-HPLC) can be employed to separate proteins because of the introduction of relatively large pore (>300 nm) columns. Pioneering work in the use of RP-HPLC to

基金项目: 国家自然科学基金(30470330)、国家自然科学基金(30470330)、浙江大学宁波理工学院青年博士基金(1050257G304)和新疆自治区科技厅自然基金项目(200121107)资助。

作者简介: 王强(1971-),浙江大学博士,多年从事作物种子科学方面的教学与研究工作。E-mail: wangqiangsky@263.net

Received(收稿日期):2003-11-28, Accepted(接受日期): 2004-04-04.

separate seed storage proteins and thereby identify cultivars and show associations of particular proteins with grain quality has been carried out by Dr. Jerold Bietz and coworkers at the USDA Northern Regional Research Center, ARS-USDA, Peoria, Illinois[4~6]. RP-HPLC has been used to characterize species and individual inbred lines or cultivars in wheat (*Triticum aestivum* L.; *T. durum* Duf.)[7], barley (*Hordeum vulgare* L.)[8], oats (*Avena* spp.)[9], and maize (*Zea mays* L.)[10], as well as for the identification of cultivated genotypes and the analysis of varietal mixtures and hybrid pedigrees.

Melon (*Cucumis melo* L.) is one of the most important fruits in China. The Xinjiang Autonomous Region in northwest China is the biggest production center of both the fresh fruit and melon seeds. In China, the planted areas of cultivars that were identified in this research totaled over 100,000 ha in 2000, while the planted areas of greenhouse-cultivated melon measured over 70 000 ha in 2001. The planted areas of the F_1 hybrid of 94 ×8-1 summed over 10 000 ha in 2002. This makes China the biggest producer of greenhouse-cultivated melon in the world (2002, CSY)[11]. The pollination characteristics of melon are open, which makes it difficult to control seed purity. The resultant seed contamination can cause heavy losses for fruit producers. Identifying inbred lines and determining F_1 hybrid purity are important quality requirements in fruit breeding and seed production. Thus, fast, simple and reliable methods for the analysis of genetic purity of melon seeds are essential for both seed suppliers and fruit growers. Therefore, the study of this paper explores the potential of RP-HPLC for the fractionation of alcohol soluble seed storage proteins from melons grown in Xinjiang and other parts of China and assesses the possibility of using this method as a means of cultivar identification.

1 Materials and Methods

1.1 Plant materials

The hybrid parents S ♂ and S ♀ and F_1 hybrids of K96-8 ×6-1, T-5 ×red1 - 28 and 94 ×8 - 1 were obtained from the Department of Horticulture at Xinjiang Academy of Agricultural Sciences. All seed lots examined were certified so that their origin and purity could be documented.

1.2 Extraction of protein from melon seeds

A single melon seed was crushed and ground with a pestle and mortar, then soaked 2 h in 0.5 mL 70 % (*V*/*V*) aqueous ethanol. The samples were then ground for a further 10 min, clarified by centrifugation (12 000 ×*g* for 10 min), and filtered through a 0.45 μm pore filter before injection into the column.

1.3 Chemicals and reagents

Unless noted otherwise all chemicals were of "Analar" grade. Acetonitrile and methol (spectra analyzed grade) were from TEDIA Company Inc., USA. Trifluoroacetic acid (spectra analyzed grade) was from British Drug Houses, London, UK. Water was doubly distilled and purified using a Waters Millipore Milli-Q purification system to a conductivity of 18 μω.

1.4 Reversed-Phase High Performance Liquid Chromatography

All chromatography data were obtained using a Shimadzu Associates Model LC 6A, Shimadzu Class VP5.0 multi-solvent delivery system and a Shimadzu SCL-6A system controller (Shimadzu, Japan). Samples were injected through a Rheodyne 7000 injection valve connected to an LKB 2157 auto sampler (LKB, Bromma, Sweden). Eluted proteins were monitored at 210 nm using a Shimadzu LC-10A TVP photodiode and ray detector (PDA). A Shimadzu CTO-6A Column oven was used to maintain constant column temperature at 60 ℃ during RP-HPLC. Column was a Waters Delta Pak C18, 5 μ particle size reversed-phase column (150 mm ×3.9 mm).

1.5 Computation

Data were acquired by means of a Shimadzu CR-3A Chromatopac, using an Intel personal computer with Nelson Analytical software that facilitated data manipulation and storage. Chromatograms of stored data were displayed on a video monitor using a Hercules graphics card. Peak areas were determined through a manual interactive integration process on the monitor by aligning the cursor at positions corresponding to the beginning and the end of an eluting peak. The area under this curve was calculated in a manner that permitted exact comparisons to be made bet ween the same time intervals for the chromatography of different samples. This ensured a reproducible calculation of peak areas. Statistical analysis of data was undertaken

using the Minitab statistical package (supplied by Minitab Inc. Pennsylvania, USA). Data in the text are expressed as Mean ± *SD*.

2 Results

2.1 Optimum separation conditions for the chromatography

Chromatographic conditions were optimized by variation in both run time and gradient conditions by using water and acetonitrile containing 0.1% (*V*/*V*) trifluoroacetic acid as solvents A and B, respectively. A linear gradient of 22% - 50% solvent B with a flow rate of 1 mL·min^{-1} and a run time of 67 min were found to give optimum separation of melon alcohol soluble proteins. Under these conditions, more than 25 different constituents could be identified in ethanol extracts from a single kernel of the cultivars. At the end of each gradient program, solvent B was increased to a final concentration of 60% over a period of 4 min before returning to initial conditions. After this, the column was treated by periodically washing with a 0 - 100% acetonitrile gradient for 15 min to remove any strongly absorbed proteins that might have accumulated on the column from run to run.

2.2 The reproducibility of column retention time for the methods

In order to test the reproducibility of column retention times for different extracts of the same cultivars, ethanol extracts of the cultivars K96-8 ×6-1 ♀ and ♂ were analyzed on different days. In our research, 94 ×8-1 ♂ samples were also analyzed on the same day. When tested on the same day, the percentage coefficient of variation for five chosen peaks (Fig. 1) was found to vary very little—average value 1.97 (Table 1). This is similar to the results of Bietz using wheat gliadins and Marchylo and Kruger using hordein proteins extracted from cultivar of Canadian barley. When tested on different days, reproducibility of retention times for the five chosen peaks (Fig. 2) tended to be more subject to variation with average coefficients of variation 4.53 and 4.56 for the two different inbreeding lines (Table 2). Comparison of elution profiles indicated that this level of reproducibility was higher than acceptable for identification of the different cultivars.

Table 1　Reproducibility of retention times (within one day) for reversed-phase separation of alcohol soluble protein (94 ×8-1 ♂)

Peak number	Mean retention time ± *SD* (min)	Coefficient of variation (%)
a	2.88 ±0.10	3.47
b	3.87 ±0.10	2.58
c	7.66 ±0.12	1.57
d	13.47 ±0.18	1.34
e	16.15 ±0.15	0.93
Average (n = 5)		1.97

Table 2　Reproducibility of retention times (from day to day) for reversed-phase separation of alcohol soluble proteins

K96-8 ×6-1 ♂			K96-8 ×6-1 ♀		
Peak number	Mean retention time ± *SD* (min)	Coefficient of variation (%)	Peak number	Mean retention time ± *SD* (min)	Coefficient of variation (%)
a	2.20 ±0.15	6.82	a	3.94 ±0.21	5.33
b	3.94 ±0.18	4.57	b	7.72 ±0.45	5.83
c	7.72 ±0.32	4.15	c	10.22 ±0.55	5.38
d	20.03 ±0.35	4.74	d	21.58 ±0.75	3.48
e	41.96 ±0.35	2.38	e	37.70 ±1.05	2.79
Average (n = 5)		4.53	Average (n = 5)		4.56

2.3 Identification results for three cultivars of melon

The RP-HPLC profiles for the hybrid parents' ♀ and ♂, and F_1 hybrids K96-8 ×6-1, T-5 ×red1-28 and 94 ×8-1 are shown in Fig. 1, 2, and 3. Repeated analyses of the extracts under similar conditions gave nearly identical elution patterns, which were characteristic for each parent. Some major qualitative differences among the resulting chromatograms will be noted. In the elution profile, characteristic peaks eluting between 0 - 12 minutes are recognizable which contain proteins common to each parent and hybrid F_1. For example, in Fig. 1, the peaks eluting at 0.60 min, 2.20 min, 2.90 min, 3.94 min, 5.73 min, 7.72 min, 35.63 min and 37.70 min represent proteins common to the hybrid parents and F_1

hybrids of K96-8 ×6-1, while the peaks eluting at 1.60 min, 32.19 min, 41.96 min and 50.69 min represent proteins common to the ♂and F_1. The peaks eluting at 8.68 min, 9.13min, 10.22 min, 13.42 min and 14.66 min represent proteins common to ♀and F_1. The peaks eluting at 47.33 min are specific to F_1 hybrids. The peaks eluting at 21.58 min, 45.74 min, 52.08 min 60.94 min and 65.17 min are specific to hybrid parent ♀, and the peaks eluting at 1.32 min, 18.53 min, 20.03 min, 23.81 min, 29.39 min and 54.86 min are specific to hybrid parent ♂. In Fig. 2, the peaks eluting at 0.55 min, 2.20 min, 2.79 min, 3.89 min, 5.83 min, 8.06 min, 8.73 min, 10.40 min, 11.26 min, and 11.93 min represent proteins common to the hybrid parents and F_1

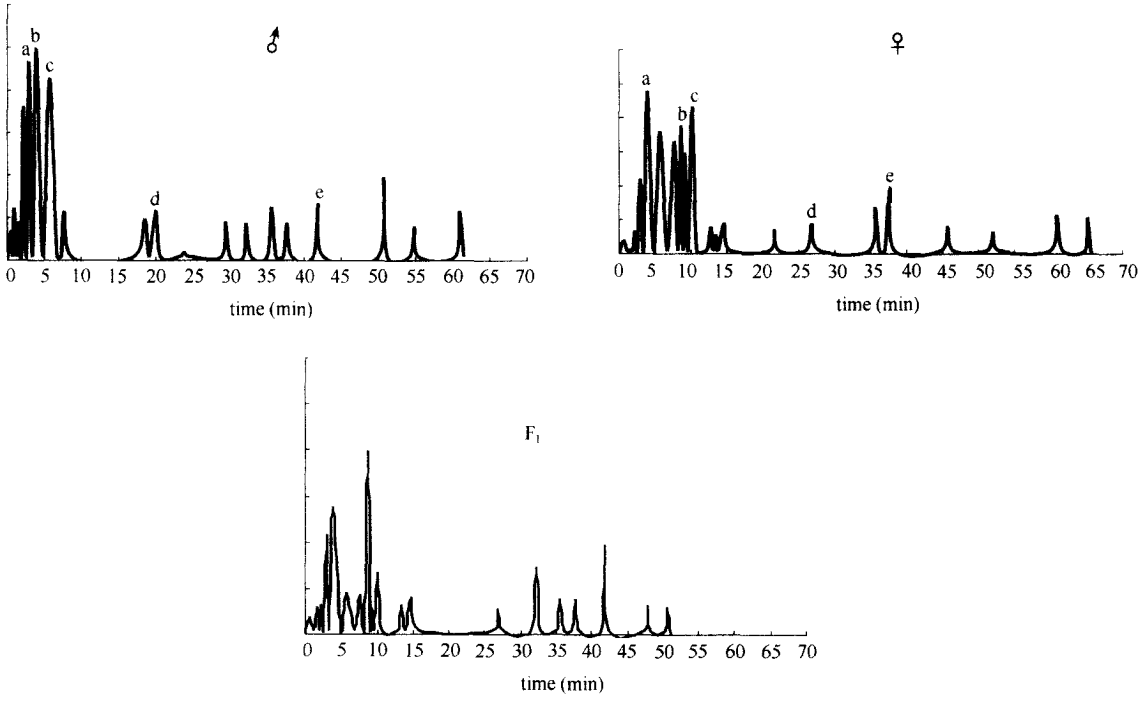

Fig. 1 RP-HPLC elution profiles of the K96-8 ×6-1 hybrid parents ♀and ♂, and F_1 hybrids

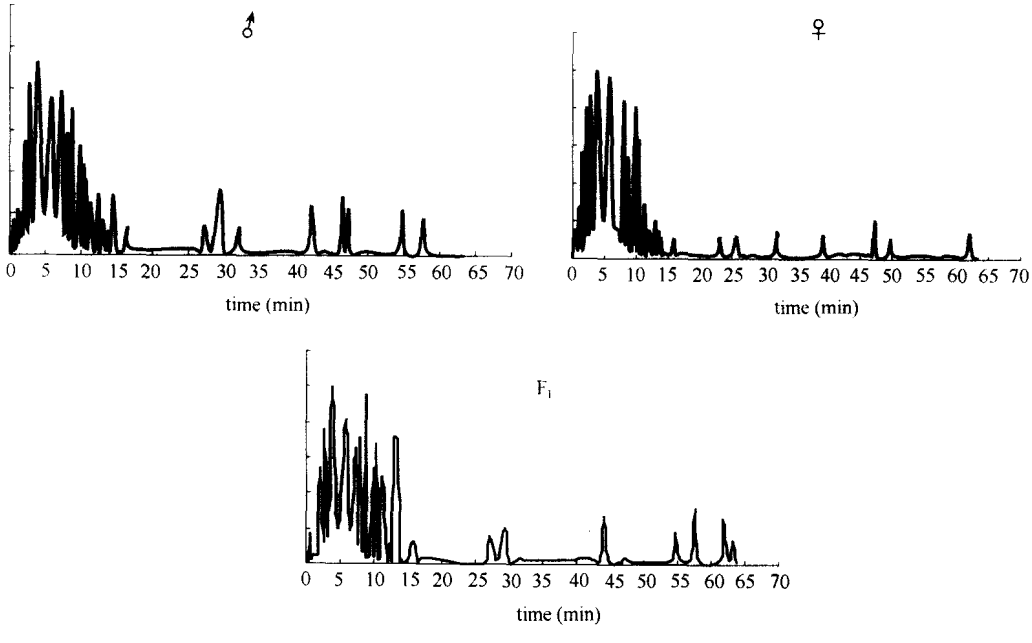

Fig. 2 RP-HPLC elution profiles of the T-5 ×red1-28 hybrid parents ♀and ♂, and F_1 hybrids

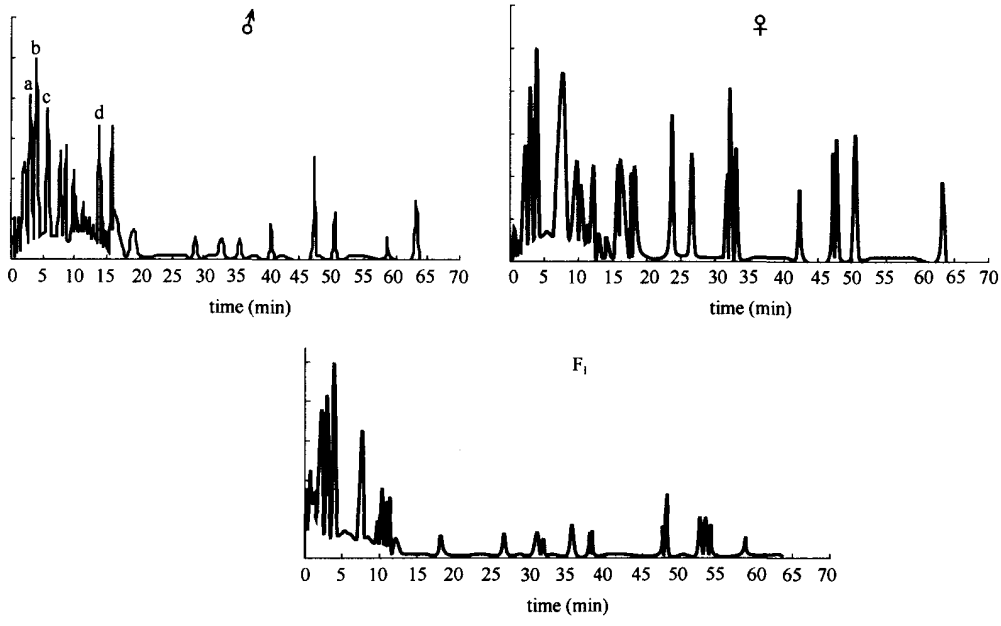

Fig. 3 RP-HPLC elution profiles of the 94 ×8-1 hybrid parents ♀ and ♂, and F_1 hybrids

hybrids 7-5 ×red1-28, while the peaks eluting at 7.25 min, 9.92 min, 12.44 min, 27.13 min, 29.41 min, 54.78 min and 57.70 min represent proteins common to the ♂ and F_1. The peaks eluting at 13.48 min and 15.94 min represent proteins common to the ♀ and F_1. The peaks eluting at 44.03 min, 61.96 min and 63.36 min represent proteins specific to F_1 hybrids, while the peak eluting at 13.84 min represents protein common to the hybrid parent. In Fig. 3, the peaks eluting at 0.70 min, 2.46 min, 2.88 min, 3.87 min, 7.66 min, 9.78 min, 10.29 min, 11.37 min and 12.69 min represent proteins common to the hybrid parents and F_1 hybrids of 94 ×8-1. The peaks eluting at 1.14 min, 10.95 min, 35.61 min and 58.78 min represent proteins common to the ♂ and F_1, and the peaks eluting at 1.42 min, 18.20 min, 26.57 min, 31.82 min and 47.69 min represent proteins common to the ♀ and F_1. The peaks eluting at 12.94 min, 14.02 min, 15.80 min, 16.15 min, 33.02 min, 50.44 min and 63.20 min represent proteins common to the hybrid parent, but not appearing in F_1 hybrids. The peaks eluting at 30.99 min, 37.98 min, 38.30 min, 52.71 min, 53.48 min and 54.21 min represent proteins appearing in F_1 hybrids but not in hybrid parents. The peaks eluting at 13.47 min, 14.79 min, 28.63 min and 40.41 min also represent proteins specific to the hybrid parent ♂. The peaks eluting at 23.68 min, 32.11 min, 42.31 min and 47.13 min represent proteins specific only to the hybrid parent ♀.

3 Discussion

The accurate description and identification of inbred lines and hybrids are prerequisite to genetic studies, as well as success in breeding and the production of both pure foundation and hybrid seeds[12]. RP-HPLC separates proteins based on the different interactions of hydrophobic sites on the surface of the protein molecule with a silicon coated column support material and a mobile organic phase that moves through the column. A mixture of proteins that are deposited onto the column support can be sequentially solublised into the organic solvent pumping through the column as the organic (hydrophobic) constitution of the solvent. Proteins of different hydrophobiuties can therefore be displaced from the column by different percentages of organic solvent. Thus, RP-HPLC yields data that is both qualitative and quantitative in describing cultivars in terms of retention times on the column and relative amounts for each protein or proteins. The method is rapid, highly reproducible and highly accurate. Data from RP-HPLC can be readily

compared and analyzed by computer.

RP-HPLC separation of gliadins has been used successfully for wheat[13,14], maize[15], rye[16] and rice[17]. Data in this research indicate that this separation technique can be used for the identification of melon cultivars. The reproducibility and resolution efficiency of RP-HPLC analysis have been used for cultivars identification in other horticultural crops such as *Brassica oleracea* L.[18] In addition to the high sensitivity and resolution of the method (HPLC theoretical plates = 10^5 vs 10^3 for SDS-PAGE), it is worth mentioning that the rapidity of analysis (67 min. ca) and the possibility of monitoring the separations allow for possible modifications in real-time. Automatic sample handling is another advantage of modern chromatographic systems. The incorporation of an automatic sampler into a system would enable sequential analyses to be run continuously. But, the disadvantages of HPLC system are: a) only one sample can be tested at one time, while SDS-PAGE allows for fifteen or more samples to be analyzed simultaneously; b) it is very difficult to determine the molecular weight and the isoelectric point of the analyzed proteins; c) it is difficult to predict their elution order.

In the light of these considerations, RP-HPLC and SDS-PAGE need not be seen as competitive methods, but as complements to each other. SDS-PAGE can be used to screen analyses because of its rapidity and simplicity, whereas RP-HPLC can be used to differentiate varieties not well characterized or indistinguishable by use of electrophoretic banding patterns.

Acknowledgements: James Gills and Samuel Kwok for their assistance with grammatical revisions.

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