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Distribution of Various Peptides in Citrus Fruits (Grapefruit, Lemon, and Orange)

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Tissue- and species-specific peptides of the grapefruit have been investigated by SDS-PAGE and Western blot. Five peptides from the juice and one peptide from the peel were isolated by preparative gel electrophoresis. Polyclonal antibodies were developed against them in mice. It can be established that 82, 63, and 46 kDa peptides occurred exclusively in the samples prepared from the grapefruit and the lemon juice, whereas in the orange juice, only the 82 kDa peptide could be detected. The 31 kDa peptide is characteristic for the peel samples of grapefruit and lemon. The 210 kDa peptide did not show any specificity. A 117 kDa peptide appeared in the juice and peel of grapefruit and in the peel of lemon but not in the orange. From the data of this study, it is supposed that some of the polyclonal antibodies developed against characteristic juice and peel peptides can be used to test commercial grapefruit juice products for adulteration.

KEYWORDS: Grapefruit; lemon; orange; peptides; polyclonal antibody; gel electrophoresis; Western blot

INTRODUCTION

A primary objective in beverage and/or juice quality control is to ensure the authenticity of the juice products and to be able to detect adulteration. The number of papers published related to adulteration of fruit juice beverages indicates the importance of this problem (*1*−*12*).

Many forms of adulteration have been found from simple dilution with water or substitution of cheap ingredients (sugar, acid, colorant essence, other types of cheap fruit juices) to sophisticated methods such as addition of byproducts (peel extract or pulp wash) to juice (*2*, *13*).

Determination of the authenticity and/or manipulation of a juice product needs specific compound(s) (*1*−*3*). Flavonoids of citrus species can be used to distinguish the orange (*Citrus sinensis*) from the grapefruit (*Citrus paradisi*) or tangerine (*Citrus reticulata*) (3, *4*, *11*, *14*). The organic acid ratio may be useful in the detection of adulteration. The ratio of malic acid/total acids or citric acid/total acids may be used as a guide for the detection of malic acid or citric acid addition to the juice. The ratios have small coefficients of variation (*2*). However, synthetic malic acid is commercially available. The ratio of isocitric acid and citric acid can be used for the detection of addition of citric acid as well (*2*, *15*−*17*). Addition of commercial isocitric acid (unstable) to the juice is expensive and can be performed only with difficulties. However, the coefficients of variation of the ratio are high, but it is used as a criterion for the detection of citric acid addition. The formol number, used as an indirect quantitative determination of free amino acids of citrus fruit products, gives information about possible dilution of beverages (*2*).

Proteins might be specific enough to characterize the various parts of citrus fruits and might be species-specific, too. Differences in protein composition of the exo-, meso-, and endocarp of citrus fruits (*18*) and species-specific immunogenicity of orange and lemon juice were reported (*19*) earlier. A method based on immunodiffusion has been worked out by Firon et al. (*20*) for the determination of the juice content in commercial products using an antiserum developed against the total protein content of the orange juice. Some tissue-specific peptides from orange were isolated and studied with SDS-PAGE and Westerns blot by Sass-Kiss and Sass (*21*).

In this work, the characteristic peptides isolated from grapefruit juice and peel have been investigated and compared with the proteins of grapefruit, lemon, and orange fruits.

MATERIALS AND METHODS

Grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), and orange (*Citrus sinensis*) were purchased at a public market. After the fruits had been peeled, the juice was obtained by a fruit juice-maker. The peel (albedo plus flavedo) was cut into small pieces.

Isolation of the Proteins from the Juice. Twenty milliliters of juice was filtered through filter paper. The filtrate was centrifuged at 15000*g* for 20 min. The sediment was resuspended in 0.5 mL of 0.5 M Tris-HCl (pH 8.3) buffer and mixed with an equal volume of Laemmli's sample buffer. The solution was boiled for 3 min. After centrifugation at 15000*g* for 15 min, the supernatant was used for SDS-PAGE.

Isolation of the Proteins from the Peel. One gram of peel was extracted in 10 mL of water (10% peel extract) and filtered through filter paper. The filtrate was centrifuged at 15000*g* for 20 min. The sediment was treated the same as that of the juice.

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Gel Electrophoresis. SDS-PAGE was carried out according to the method of Laemmli (*22*) on a 13.6% w/v separation gel overlaid by 3% stacking gel in a Bio-Rad Mini Gel Chamber. Gels were stained by Coomassie BBR. Two microliters from the juice samples and 7 μL from the peel samples were loaded on the gel. The concentration of protein samples of juice and peel extract were 29 and 5 mg/mL, respectively (Bradford method).

Separation of Peptides by Preparative Gel Electrophoresis. Preparative SDS−polyacrylamide gels (1.5 mm thick) were used to separate the peptides of the sample and the standard, respectively. After electrophoresis, two edges of the gel were cut and stained to determine the position of the peptides of interest, which were then cut from the gels. Peptides were electroeluted into dialysis bags (cellulose membrane retaining proteins with MW 12400) in a Mini Trans Blot chamber (Bio-Rad) for 600 V·h. The peptide samples (∼6 mL) were dialyzed overnight against distilled water at 4 °C and lyophilized. The peptides were redissolved in 60−100 μL of final volume. The purity of peptides was tested by analytical SDS-PAGE, and the procedure was repeated again to obtain electrophoretically homogeneous samples.

Preparation of Antibodies. Isolated peptides (25−30 μg) in 100 μL of PBS were injected with an equal volume of Freund's complete adjuvant into the skin of male BALB/c mice. Animals were boosted 3 weeks later with the same amount of protein emulsified in 100 μL of Freund's incomplete adjuvant. The titer of antibodies was tested in blood samples collected from the tip of the tail of the mice. The third booster was normally enough to achieve a useful titer of antibodies against our peptides. After the immunizations had been completed, animals were bled and the IgG fraction was separated from the sera by ammonium sulfate precipitation.

Immunoblotting. Peptides were transferred from SDS−polyacrylamide gel to a sheet of nitrocellulose filter (Bio-Rad, 0.2 μm pore size) by electroblotting according to the method of Towbin et al. (*23*). The nonspecific binding sites were blocked by 5% Carnation nonfat dry milk powder in TBS buffer (0.15 M Tris-HCl, 0.5 M NaCl, pH 7.0). After three washings with TTBS buffer (TBS buffer containing 0.05% Tween 20), the nitrocellulose sheet was incubated in the presence of the first antibody, diluted in TBS for 12 h at 4 °C. The blot was washed three times in TTBS and incubated with the second antibody (alkaline phosphatase labeled anti-mouse antibody, Bio-Rad) dissolved in TBS in 1:1000 for 1 h at room temperature. After the final washes, the blot was developed in the solution of freshly prepared substrate (BCIP and NBT, Bio-Rad). Molecular masses of the positive bands were determined using Bio-Rad prestained standards (myosin, 206 kDa; β-galactosidase, 120 kDa; bovine serum albumin, 84 kDa; ovalbumin, 52 kDa; carbonic anhydrase, 36 kDa; soybean trypsin inhibitor, 30 kDa; lysozyme, 22 kDa; aprotinin, 7.5 kDa).

RESULTS AND DISCUSSION

The protein patterns of the juice and peel samples prepared from the grapefruit were analyzed by SDS-PAGE. On the gels, the characteristic peptides of the juice and the peel were identified (**Figure 1**.) The numbers displayed in the figure show the molecular masses of the specific peptides in the juice and peel. The results were in agreement with our earlier observation (*21*) when the proteins were isolated after precipitation of proteins with trichloroacetic acid (TCA). The presence of three more characteristic peptides (82, 52, and 46 kDa) was detected in the juice in present study.

Isolation of the peptides was preformed by preparative gel electrophoresis. The purity of the isolated peptides was tested by SDS-PAGE. The electrophoretogram of peptides prepared from grapefruit juice having molecular masses of 210, 117−87, 82, 65, 46, and 31 kDa from the grapefruit peel is shown in **Figure 2**.

Polyclonal antibodies were developed against isolated peptides in mice, and their purity was tested on Western blots. The presence and tissue-specific distribution of the antigenes in grapefruit, lemon, and orange have been studied by Western blots as well (*23*).

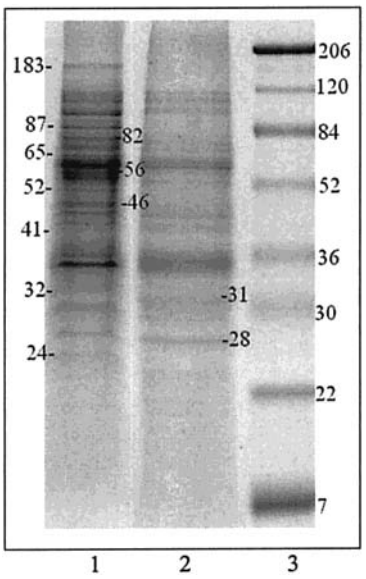

Figure 1. Gel electrophoretogram of peptide samples prepared from grapefruit juice and grapefruit peel: (1) grapefruit juice; (2) grapefruit peel; (3) molecular mass standard (Bio-Rad).

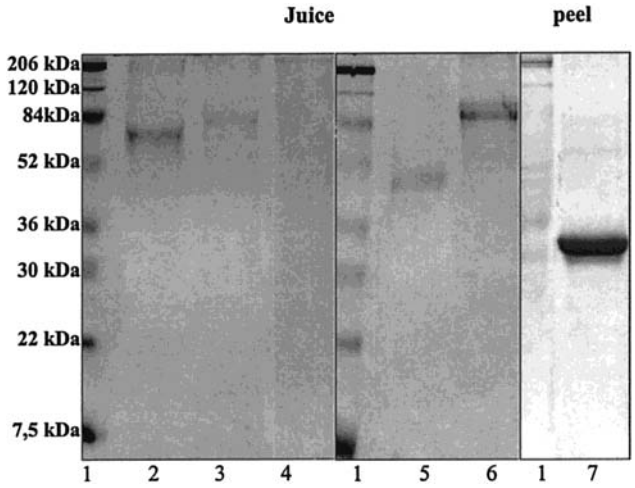

Figure 2. Gel electrophoretogram of isolated peptides from grapefruit juice and peel: (1) molecular mass standard (Bio-Rad): (2) 65 kDa peptide; (3) 82 kDa peptide; (4) 210 kDa peptide; (5) 46 kDa peptide; (6) 117−87 kDa peptide; (7) 31 kDa peptide.

Figure 3 shows the Western blot of juice and peel samples of grapefruit, lemon, and orange using nonimmune mouse serum as negative control.

Western blots stained by polyclonal antibodies developed against isolated peptides of molecular mass of 117−87 kDa are provided in **Figure 4**.

On the blot, the polyclonal antibody developed against the 117 kDa peptide was used in 500-fold dilution. It gave a positive reaction with a single band of 117 kDa peptide in the juice and peel samples of the grapefruit. In lemon samples, the antibody gave a positive reaction with the 117 kDa band of peel but did not react with any peptides of the lemon juice. The orange did not give immune reaction with this antibody.

Figure 5 shows the Western blot of polyclonal antibody developed against the 82 kDa peptide isolated from grapefruit juice. The polyclonal antibody was tested with protein samples containing all of the peptides prepared from grapefruit, lemon, and orange. During the blotting procedure, the antibodies were used in 500-fold dilution. The antibody gave positive reaction

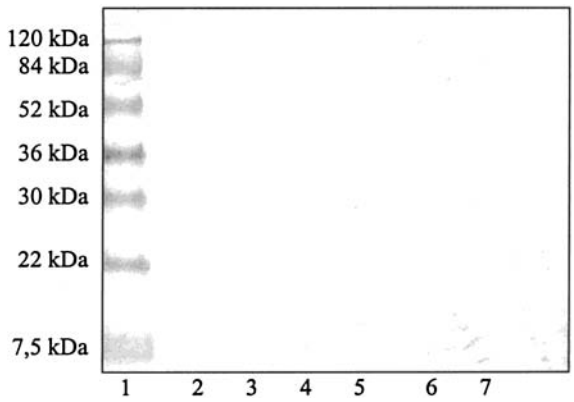

Figure 3. Western blot with nonimmune mice serum: (1) molecular mass standard; (2) grapefruit peel, (3) grapefruit juice; (4) lemon peel; (5) lemon juice; (6) orange peel; (7) orange juice.

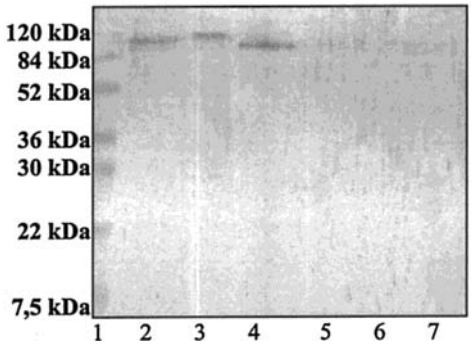

Figure 4. Western blot with antibody (500-fold dilution) developed against 117 kDa peptide isolated from grapefruit juice: (1) molecular mass standard; (2) grapefruit peel; (3) grapefruit juice; (4) lemon peel; (5) lemon juice; (6) orange peel; (7) orange juice.

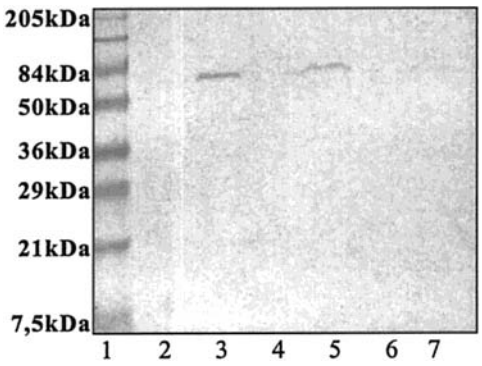

Figure 5. Western blots of citrus juice and peel samples with polyclonal antibody (500-fold dilution) developed against peptide of molecular mass of 82 kDa isolated from grapefruit juice: (1) molecular mass standard; (2) grapefruit peel; (3) grapefruit juice; (4) lemon peel; (5) lemon juice; (6) orange peel; (7) orange juice.

with a single peptide band of 82 kDa of grapefruit, lemon, and orange juice on the blot. In the orange juice, this peptide band could be just detected. The antibody did not give immunochemical cross-reaction with the peel samples of grapefruit, lemon, and orange. On the basis of these results it can be established that the 82 kDa peptide is a juice-specific one characterizing only the juice and not the peel of the three studied citrus fruits.

From the grapefruit peel, a polyclonal antibody was developed against the 31 kDa peptide. The blot using this antibody (15000-fold dilution) can be seen in **Figure 6**. The antibody gave immune reaction with the 31 kDa peptide of the peel of the

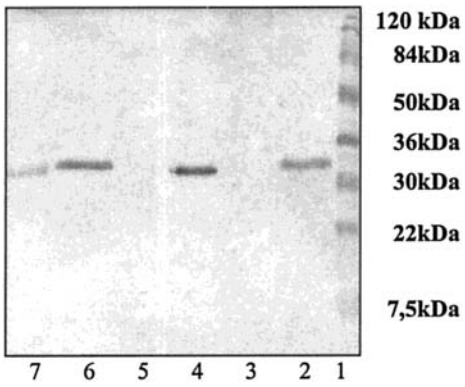

Figure 6. Western blot of citrus juice and peel samples with polyclonal antibody (15000-fold dilution) developed against 31 kDa peptide isolated from grapefruit peel: (1) grapefruit peel; (2) grapefruit juice; (3) lemon peel; (4) lemon juice; (5) orange peel; (6) orange juice; (7) molecular mass standard.

Table 1. Distribution of Isolated Peptides in Various Parts of Citrus Fruits

polyclonal antibody of peptide	dilution of antibody	orange		grapefruit		lemon	
		juice	peel	juice	peel	juice	peel
210 kDa	500-fold	+	+	+	+	+	+
117 kda	500-fold			+	+		+
82 kDa	500-fold	(+)[a]		+		+	
65 kDa	250-fold			+		+	
46 kDa	250-fold			+		+	
31 kDa	15000-fold	(+)	+		+		+

[a] (+), concentration of the peptide is low.

grapefruit, lemon, and the orange, whereas in the grapefruit and lemon juice samples, no peptide bands appeared on the blot. In orange juice a very pale peptide band could be detected. According to the results, the 31 kDa peptide occurred exclusively in the grapefruit and lemon peel but not in the juice.

Using antibodies developed against all isolated peptides, the distribution of the antigens in the various parts of the citrus fruits is summarized in **Table 1**.

On the basis of these results we established that we could develop antibodies against three juice-specific peptides as the 82 kDa peptide occurred in the juice of orange, grapefruit, and lemon and the 65 and 46 kDa peptides occurred exclusively in juice of grapefruit and lemon. The 31 kDa peel peptide is characteristic for the peel samples of grapefruit and lemon but not specific for orange peel. The 210 kDa peptide was not a specific one because it appeared in the juice and the peel of all studied citrus fruits. The 117 kDa peptide did not show tissue specificity in grapefruit and could not be detected in orange samples but occurred exclusively in the lemon peel.

Two antibodies of the above were used for testing of commercial grapefruit juice products. One of the juice product was a 100% processed grapefruit juice, and the other one was a 40% red grapefruit nectar. In **Figure 7a**, the blot shows the immune reaction of the antibody developed against the juice peptide of 82 kDa. In **Figure 7b**, the immune reaction of the antibody developed against the peel peptide of 31 kDa is demonstrated.

The antibody of the 82 kDa peptide gave a single positive band with the peptides prepared from commercial grapefruit juice products. The peptide bands were less intense in processed grapefruit juice than in the laboratory grapefruit samples, presumably due to the processing technology of the juice products.

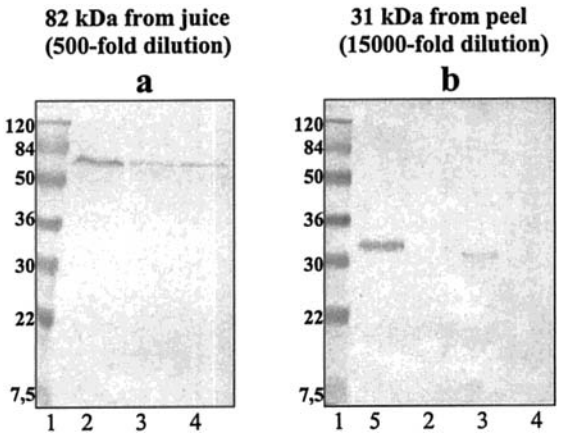

Figure 7. Western blots of (a) grapefruit juice products with antibodies developed against 82 kDa peptide isolated from grapefruit juice and (b) 31 kDa peptide isolated from grapefruit peel: (1) molecular mass standard; (2) grapefruit juice; (3); 40% grapefruit nectar with scarcocarb processed; (4) 100% grapefruit juice processed; (5) grapefruit peel.

On the blot stained with the antibody developed against the 31 kDa peel peptide, a single positive reaction band could be observed with the grapefruit peel peptides prepared in the laboratory. There were no reactions with a sample of the juice. One of the commercial juice products declared as 100% grapefruit juice did not give a positive reaction with this antibody, whereas a pale but well visible band appeared in the peptide sample of the 40% red grapefruit juice nectar. (In the specification, it was indicated on the box of the grapefruit nectar that the nectar contained fruit flesh as well.) These results indicate that the antibodies could detect other components of the fruit in the juice. The data confirm that addition of grapefruit byproducts to grapefruit juice can be detected with the antibody of the 31 kDa peptide.

In our study, four tissue-specific peptides were isolated from grapefruit juice and peel. One juice peptide (82 kDa) and one peel peptide (31 kDa) occurred in all investigated citrus fruits. Two of the juice peptides (65 and 46 kDa) did not appear in the orange juice. A species-specific peptide appearing only in grapefruit could not be isolated. Antibodies developed against juice and peel peptides can be used to detect these peptides from citrus juice and peel samples prepared in the laboratory. We suppose that this would hold true for processed juice products as well. There is the possibility to develop a quantitative (ELISA) method for determining the juice content of citrus juice products using these antibodies. Unfortunately, we could not isolate any peptides of grapefruit showing species specificity during the present study, although it would be important as well.

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