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Determination of Chitinase Activity in Different Plant Samples of Turkey

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The chitinase activity constitutively present in fruits, vegetables and cereal grains from various healthy plant species growing in Turkey have been determined. Crude extracts from 47 different fruit species, 35 kind vegetables and 4 various cereal grains were tested for chitinase activity using colloidal chitin as a substrat. In general, fruits seem to be the best sources of chitinase activity. The highest activity was found in persimmon fruits and grape berries.

Key Words: Chitinase activity, Fruit, Vegetable, Cereal.

INTRODUCTION

Chitinases (Chitin glycanohydrolase, EC 3.12.1.14) catalyze the hydrolysis of chitin, an insoluble linear homopolymer of β-1,4-linked N-acetylglucosamine (GlcNAc) residues[1]. Chitin is the second most abundant organic compound after cellulose and present in the walls of higher fungi, in the exoskeletons of insects, arachnids and many other groups of invertebrates and as an extracellular polymer of some bacteria[2]. All organisms which contain chitin also contain chitinases which are required for morphogenesis of cell walls and exoskeletons. Other organisms that do not contain chitin may produce chitinases to degrade the polymer for food, *e.g.* soil bacteria that secrete chitinases in response to chitin in their environment and the digestive tract of fish. Plant do not contain chitin in their cell walls[3,4]. However, chitinases are generally expressed in plants are used for self-defence against plant pathogens and pests[5-7]. Higher plants induce a series of proteins in response to the infection by pathogens. These proteins are called pathogenesis-related proteins (PR-proteins). Chitinase is considered to be a defence related protein in higher plants and to protect plants against fungal pathogens by degrading chitin, a major component of the cell walls of many fungi[8,9]. By rapidly and fully degrading an important

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cell wall constituent, the combination of endochitinases and exochitinases may be more effective than either of the two enzymes alone[10]. The chitinases from several species of plants have been shown to be comprised of the acidic and basic isoforms. The acidic and basic isoforms of chitinase are induced in plants in response to pathogen attack and other environmental stimuli and are also expressed in certain tissues of plants during normal development[11].

The expression of chitinase genes is regulated in a complex manner in plants. In most plants, acidic and basic isoforms of chitinase are encoded by multigene families. These genes are differentially expressed during development and are induced by a variety of defence-related and environmental stimuli. The acidic and basic isoforms of chitinases also display distinct patterns of subcellular localization. Basic isoforms of the chitinases are located predominantly in vacuoles, whereas acidic isoforms are secreted into the extracellular compartment.

Synthesis of plant chitinases can be induced not only pathogen stimulation but also wounding, heat shock, ultraviolet light, the phyto-hormone ethylene, fungal cell-wall hydrolyzate and chemicals such as salicylic acid, mercuric chloride and lead nitrate. Chitinases are widely distributed throughout the plant kingdom, either in stems and leaves following induction by ethylene or pathogen attack, or stored in seeds as a means of increasing the seeds resistance to fungi in the soil[12-14]. Most plants possess a number of chitinase isozymes that differ in primary structure, molecular weight, isoelectric point and subcellular localization[15].

In this study, we determined the chitinase activity constitutively present in 47 fruits, 35 kind vegetables and 4 cereal grains from various healthy plant species growing in Turkey.

EXPERIMENTAL

Substrates: β-D-N-acetylglucosamine was obtained from Sigma. Colloidal chitin was prepared according to the following procedure[16]. Chitin coarse flakes from crab shells were purchased from Sigma. The other reagents used were of the highest grade commercially available.

Preparation of colloidal chitin: 10 g of chitin was mixed with 500 mL of 85% phosphoric acid and stirred for 24 h at 4ºC. The suspension was poured into 5 L of deionized water and centrifuged (12000 rpm for 10 min). The resulting precipitate was washed with deionized water until the pH reached 5.0 and then neutralized by addition of 6 N NaOH. The suspension was centrifuged (12000 rpm for 10 min) and washed with 3 L of deionized water for desalting. The resulting precipitate was suspended with deionized water. The chitin content in the suspension was determined by drying a sample (final concentration, 1 %)[16].

Homogenization: Fruits, vegetables and cereal grains were obtained from local market of Istanbul (Turkey). The samples were washed with distilled water, dried in filter paper. All homogenization steps were carried out at 4ºC. Samples (2.5 g) were homogenized with 25 mL of 0.1 M citrate buffer, pH 5 in a blender. After straining through two layers of cheesecloth, the filtrate was centrifuged at 10000 rpm for 0.5 h at 4ºC. The supernatant was used as enzyme source. Five different samples from each fruits, vegetables and cereal grains were tested for enzyme assay and protein determination.

Enzyme assay: Chitinase was assayed by a modified method of Miller[17] with colloidal chitin as the substrate. The standard asay was performed at 50ºC in 0.1 M citrate buffer (pH 5.0) for 0.5 h. The reaction was terminated by adding 2.5 M NaOH and the amount of reducing sugar generated was measured. One unit of chitinase activity was defined as the amount of enzyme which produced 1 μmol of reducing sugar per min. Specific activity was expressed as units per mg of protein.

Protein measurement: Protein was measured by the method of Lowry[18] using bovine serum albumin as the standard.

Statistical analysis: The results were evaluated using by analysis of varience (ANOVA) using the NCSS statistical computer package[19].

RESULTS AND DISCUSSION

Fruit extracts of various species were analyzed for chitinase activity. Fruits exhibited the highest activity while vegetable and cereal grain extracts showed lower activity. Persimmon fruit and grape berries were showed the highest specific activity. All fruit samples tested represented significant amount of chitinase (Table-1).

Chitinase activity was also investigated in vegetables. Tomato and carrot exhibited the highest activity for vegetables. However, celery root, purslane, collard greens and pea seeds did not show chitinase activity. An explanation could be the possibility that these extracts could contain chitinase inhibitors or proteases active on chitinases (Table-2).

Four species belonging to three families were tested for cereal grains. Overall, the lowest activity was found in cereal grain extracts rather than in fruit and vegetable extracts. When considering the amount of protein in the different extracts, the highest chitinase activities are not necessarily related to the total amount of protein (Table-3).

Our results are concluded that fruits, which are rich in sugars exhibit higher chitinase activity than others. It has been reported that levels of chitinase were higher in pumpkin (*Cucurbita pepo* L.) fruit than in leaves, stems or seeds. Derckel *et al.*[20] reported that chitinase activity in grape (*Vitis vinifera* L.) berries was also higher than in leaves, roots or stems.

TABLE-1
CHITINASE ACTIVITY AND PROTEIN AMOUNT IN VARIOUS FRUIT EXTRACTS

Fruits	Family	Total protein amount (mg/mL)	Total unit (U/mL)	Specific activity (U/mg protein)
Grape berries-Sultana	*Vitaceae*	0.64 ± 0.08	2.14 ± 0.140	3.39 ± 0.24
Grape berries-Green	*Vitaceae*	0.65 ± 0.23	1.66 ± 0.110	2.75 ± 0.78
Grape berries-Red	*Vitaceae*	1.03 ± 0.39	1.63 ± 0.560	1.64 ± 0.38
Medlar	*Rosaceae*	0.94 ± 0.72	1.58 ± 0.540	2.92 ± 2.65
Persimmon fruit	*Ebeneceae*	0.40 ± 0.16	1.36 ± 0.110	4.16 ± 2.39
White mulberry	*Moraceae*	1.02 ± 0.32	1.11 ± 0.340	1.10 ± 0.26
Fig-black	*Moraceae*	0.89 ± 0.28	1.28 ± 0.260	1.51 ± 0.29
Pomegranate	*Lythraceae*	0.73 ± 0.27	1.26 ± 0.350	1.89 ± 0.58
Fig	*Moraceae*	0.88 ± 0.11	1.21 ± 0.170	1.38 ± 0.26
Amasya apple	*Rosaceae*	0.56 ± 0.06	1.18 ± 0.140	2.14 ± 0.16
Blackberry	*Rosaceae*	1.33 ± 0.25	1.17 ± 0.580	0.84 ± 0.32
Sour cherry	*Rosaceae*	1.17 ± 0.25	1.12 ± 0.150	0.98 ± 0.17
Cherry	*Rosaceae*	1.24 ± 0.36	1.10 ± 0.400	0.90 ± 0.30
Apple-red	*Rosaceae*	0.81 ± 0.58	1.10 ± 0.450	1.54 ± 0.54
Apple-yellow	*Rosaceae*	0.69 ± 0.29	1.08 ± 0.140	1.78 ± 0.74
Quince	*Rosaceae*	0.62 ± 0.37	0.91 ± 0.210	1.80 ± 0.88
Cornelian cherry	*Rosaceae*	0.82 ± 0.19	0.87 ± 0.160	1.10 ± 0.29
Pear-yellow	*Rosaceae*	0.54 ± 0.10	0.87 ± 0.310	1.71 ± 0.75
Black mulberry	*Moraceae*	1.02 ± 0.22	0.82 ± 0.120	1.01 ± 0.38
Kiwifruit	*Actinidiaceae*	0.97 ± 0.44	0.77 ± 0.420	0.78 ± 0.23
Orange bark	*Rutaceae*	2.19 ± 0.28	0.77 ± 0.140	0.36 ± 0.07
Ankara pear	*Rosaceae*	0.42 ± 0.02	0.77 ± 0.360	1.87 ± 0.96
Turkish banana	*Musaceae*	0.70 ± 0.08	0.74 ± 0.060	1.06 ± 0.15
Raspberry	*Rosaceae*	1.15 ± 0.80	0.71 ± 0.260	0.79 ± 0.39
Banana	*Musaceae*	0.53 ± 0.17	0.66 ± 0.280	1.22 ± 0.32
Watermelon	*Cucurbitaceae*	0.23 ± 0.11	0.59 ± 0.100	2.93 ± 1.20
Orange	*Rutaceae*	0.72 ± 0.09	0.58 ± 0.190	0.83 ± 0.37
Japanese medlar	*Rosaceae*	2.12 ± 1.34	0.58 ± 0.240	0.54 ± 0.26
Grapefruit bark	*Rutaceae*	4.16 ± 1.42	0.54 ± 0.170	0.13 ± 0.04
Strawberry	*Rosaceae*	0.64 ± 0.26	0.49 ± 0.140	0.86 ± 0.38
Grapefruit	*Rutaceae*	0.76 ± 0.12	0.47 ± 0.190	0.60 ± 0.22
Apricot	*Rosaceae*	0.32 ± 0.08	0.30 ± 0.240	1.00 ± 0.90
Peach	*Rosaceae*	0.38 ± 0.06	0.36 ± 0.020	1.02 ± 0.28
Mandarin	*Rutaceae*	0.60 ± 0.13	0.35 ± 0.050	0.63 ± 0.24
Greengage plum	*Rosaceae*	0.26 ± 0.08	0.33 ± 0.070	1.36 ± 0.45
Bluebyrd plum	*Rosaceae*	0.43 ± 0.10	0.31 ± 0.020	0.74 ± 0.15
Cherry plum	*Rosaceae*	0.40 ± 0.07	0.16 ± 0.080	0.39 ± 0.18
Lemon	*Rutaceae*	0.54 ± 0.09	0.03 ± 0.004	0.07 ± 0.01

TABLE-2
CHITINASE ACTIVITY AND PROTEIN AMOUNT IN VARIOUS VEGETABLE EXTRACTS

Vegetables	Family	Total protein amount (mg/mL)	Total unit (U/mL)	Specific activity (U/mg protein)
Onion	*Alliaceae*	0.73 ± 0.20	0.50 ± 0.060	0.71 ± 0.110
Carrot	*Apiaceae*	0.24 ± 0.02	0.37 ± 0.030	1.56 ± 0.120
Cabbage-red	*Brassicaceae*	1.22 ± 0.20	0.36 ± 0.010	0.30 ± 0.060
Tomato	*Solanaceae*	0.20 ± 0.08	0.36 ± 0.110	1.93 ± 0.920
Leek	*Alliaceae*	0.35 ± 0.10	0.33 ± 0.200	0.90 ± 0.260
Squash	*Cucurbitaceae*	0.42 ± 0.06	0.29 ± 0.040	0.68 ± 0.140
Eggplant	*Solanaceae*	0.49 ± 0.17	0.28 ± 0.090	0.68 ± 0.500
Onion-aerial part	*Alliaceae*	0.45 ± 0.04	0.27 ± 0.040	0.61 ± 0.120
Cabbage	*Brassicaceae*	0.20 ± 0.06	0.27 ± 0.150	1.56 ± 1.280
Pea pod	*Fabaceae*	0.58 ± 0.35	0.27 ± 0.140	0.48 ± 0.050
Garlic-aerial part	*Alliaceae*	0.83 ± 0.32	0.26 ± 0.160	0.45 ± 0.120
Horse-bean pods	*Fabaceae*	2.76 ± 1.35	0.26 ± 0.060	0.11 ± 0.050
Chile pepper	*Solanaceae*	0.32 ± 0.09	0.26 ± 0.060	0.80 ± 0.040
Cayenne pepper	*Solanaceae*	0.70 ± 0.26	0.25 ± 0.110	0.35 ± 0.030
Green beans	*Fabaceae*	0.28 ± 0.06	0.23 ± 0.050	0.85 ± 0.210
Cauliflower	*Brassicaceae*	0.35 ± 0.06	0.22 ± 0.110	0.65 ± 0.320
Radish	*Brassicaceae*	0.43 ± 0.06	0.22 ± 0.110	0.52 ± 0.320
Olive berries	*Oleaceae*	4.64 ± 0.49	0.20 ± 0.100	0.04 ± 0.020
Iceberg lettuce	*Asteraceae*	0.40 ± 0.16	0.17 ± 0.030	0.56 ± 0.450
Lettuce	*Asteraceae*	0.40 ± 0.18	0.18 ± 0.060	0.28 ± 0.230
Cucumber	*Cucurbitaceae*	0.19 ± 0.07	0.13 ± 0.040	0.76 ± 0.490
Cos lettuce	*Asteraceae*	0.25 ± 0.02	0.12 ± 0.060	0.49 ± 0.280
Maize	*Poaceae*	0.37 ± 0.02	0.12 ± 0.030	0.32 ± 0.060
Garlic	*Alliaceae*	2.02 ± 0.32	0.07 ± 0.013	0.03 ± 0.006
Horse-bean seed	*Fabaceae*	1.71 ± 0.64	0.07 ± 0.040	0.04 ± 0.010
Parsley	*Apiaceae*	0.52 ± 0.14	0.06 ± 0.020	0.12 ± 0.050
Okra	*Malvaceae*	0.66 ± 0.15	0.04 ± 0.008	0.06 ± 0.010
Chard	*Amaranthaceae*	0.71 ± 0.50	0.03 ± 0.010	0.06 ± 0.008
Potatoes	*Solanaceae*	0.75 ± 0.22	0.03 ± 0.005	0.05 ± 0.003
Spinach	*Amaranthaceae*	0.92 ± 0.20	0.03 ± 0.007	0.04 ± 0.010
Nettle	*Urticaceae*	1.44 ± 0.26	0.03 ± 0.010	0.02 ± 0.005
Celery root	*Apiaceae*	0.40 ± 0.09	–	–
Purslane	*Portulacaceae*	0.50 ± 0.06	–	–
Collard greens	*Brassicaceae*	0.94 ± 0.12	–	–
Pea	*Fabaceae*	0.86 ± 0.06	–	–

TABLE-3
CHITINASE ACTIVITY AND PROTEIN AMOUNT IN VARIOUS DRIED FRUITS, NUTS AND CEREAL GRAIN EXTRACTS

Cereal grains	Family	Total protein amount (mg/mL)	Total unit (U/mL)	Specific activity (U/mg protein)
Dried grape berries-Sultana	*Vitaceae*	2.38 ± 0.38	4.70 ± 1.800	2.09 ± 1.110
Dried fig	*Moraceae*	1.72 ± 0.08	4.70 ± 3.020	2.79 ± 1.940
Dried white mulberry	*Moraceae*	2.35 ± 0.56	3.92 ± 0.200	1.73 ± 0.410
Dried apricot	*Rosaceae*	2.04 ± 0.68	3.01 ± 2.400	1.59 ± 1.340
Carob pods	*Fabaceae*	2.04 ± 0.32	0.93 ± 0.260	0.46 ± 0.140
Apricot seed	*Rosaceae*	5.29 ± 1.26	0.35 ± 0.270	0.06 ± 0.040
Walnut	*Juglandaceae*	4.82 ± 1.62	0.24 ± 0.120	0.06 ± 0.030
Pumpkin seed	*Cucurbitaceae*	3.45 ± 2.34	0.11 ± 0.020	0.02 ± 0.001
Hazelnut	*Betulaceae*	3.80 ± 1.16	0.09 ± 0.060	0.02 ± 0.010
Lentil-green	*Fabaceae*	2.36 ± 0.04	0.06 ± 0.008	0.02 ± 0.004
Haricot bean	*Fabaceae*	2.69 ± 1.28	0.03 ± 0.010	0.01 ± 0.000
Lentil-red	*Fabaceae*	2.36 ± 0.18	0.03 ± 0.003	0.01 ± 0.000
Rice	*Poaceae*	0.22 ± 0.05	0.02 ± 0.010	0.09 ± 0.060

Fruits provide an ideal target for pathogens. Ripening of fruit involves the accumulation of sugars and other nutrients as well as softening and eventual breakdown of cellular structure, all of which would predispose the tissue toward a pathogen attack. A number of defence mechanisms developed in germinating seeds and in floral tissues. They also provide excellent targets for pathogens and it may be that a similar situation occurs in ripening fruit. Since the tissue is likely to become highly susceptible to pathogens, induction of pathogen related proteins may develop rather than in response to a pathogen attack. Alternatively, the constitutive expression of protein related proteins in these tissues may not be related to pathogen resistance, but these proteins may have a role in normal growth and development[21].

Plants evolved a number of strategies to resist fungal infection. One strategy involves the accumulation of defence proteins that have direct inhibitory activity against the hyphae and/or germinating spores of the pathogen. Another physiological adaptation of plants that affects fungal pathogenesis, is the accumulation of sugars. The interaction of the antifungal proteins and sugars appears to constitute a developed regulated defence mechanism to restrict fungal pathogen infection. At least two potentially interrelated mechanisms may be involved in the regulation of antifungal protein acumulation during ripening: osmotic due to sugar accumulation, or sugar signaling. Sugars may act as a signal molecule to

regulate the expression of antifungal protein genes during fruit ripening of fruits in general. Sugars are also well known to stabilize proteins against denaturation. At physiological temperatures, proteins are preferentially hydrated. Sugars and polyhydric alcohols, are preferentially excluded from the hydration surface, a process that requires free energy. Unfolding of proteins is energetically unfavourable because solute exclusion would be required for a larger surface area. Presumably, the sugars in ripening fruits would enhance the function of the antifungal proteins by facilitating active conformation in the physiological solution. A chemical basis for sugar enhancement of antifungal activity is very plausible.

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