



# Asian Journal of Plant Sciences

ISSN 1682-3974

**science**  
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## Analysis of Free Amino Acid and Total Protein Content in Pollen of Some Allergenic Taxa

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**Abstract:** This study reports the free amino acid content of pollen grains obtained from old and fresh samples belonging to *Pinus nigra* subsp. *nigra* var. *caramanica* (Loudon) Rehder (black pine) (Pinaceae), *Juglans regia* L. (walnut) (Juglandaceae), *Fraxinus angustifolia* Vahl. (ash) (Oleaceae) and *Betula pendula* L. (birch) (Betulaceae) obtained with the technique of Liquid Chromatography-Mass Spectrometry (LC/MS). Pollen samples were obtained from flowers of the above mentioned taxa in different years. Twenty one amino acids were identified. No histidine was found in *F. angustifolia* collected 8 years ago. Total protein content of *P. nigra* subsp. *nigra* var. *caramanica* pollens (25.75%) was higher than the remaining taxa; *F. angustifolia* (13.67%), *B. pendula* (7.73%) and *J. regia* (7.55%).

**Key words:** Amino acids, LC/MS, pollen grain, *P. nigra* subsp. *nigra* var. *caramanica*, *Juglans regia*, *Fraxinus angustifolia*, *Betula pendula*

### INTRODUCTION

Pollen grains of *P. nigra* subsp. *nigra* var. *caramanica* (Loudon) Rehder, *Juglans regia* L., *Fraxinus angustifolia* Vahl. and *Betula pendula* L. are considered to be important allergenic tree pollens. These taxa are commonly cultivated in parks, recreation areas and waysides in Ankara. The flowering period for *P. nigra* subsp. *nigra* var. *caramanica* is May and June, for *J. regia* and *F. angustifolia* in April and May, for *B. pendula* in April (Inceoğlu *et al.*, 1994; Doğan and Erik, 1995). They produce copious amounts of pollen grains that can linger in stagnant air for day. It is well known that a number of people suffer from allergy and respiratory diseases which are caused by these pollen grains of the above mentioned taxa (Vik *et al.*, 1987; Aytuğ *et al.*, 1990; Nilsson and Spiekma, 1994; Mondal *et al.*, 1998a; Schappi *et al.*, 1999; Belmonte *et al.*, 2000; Burge and Rogers, 2000; Pehlivan *et al.*, 2003; Holmquist *et al.*, 2005). Pollen allergy is caused by proteins, glycoproteins or even single peptides present in the pollen wall and cytoplasm (Mondal *et al.*, 1998a). Pollen proteins are stored in the pollen wall layers (exine and intine) (Knox and Heslop-Harrison, 1970). Recent developments in pollen chemistry have improved the means to determine which proteins or amino acids cause allergic reactions in the body (Vik *et al.*, 1987; Karnakar and Chatterjee, 1992;

Parui and Mandal, 1998; Vega-Maray *et al.*, 2006; Shahali *et al.*, 2007; Russell *et al.*, 2008). According to Stanley and Linskens (1974), total levels of free amino acids in pollen grains are higher than other plant tissues.

The present study deals with the analysis of the free amino acid and total protein content of some allergenic taxa. The obtained results are expected to be functional and useful for physicians.

### MATERIALS AND METHODS

Pollen grains were collected in different stages of inflorescence in 2000 and in 2008 from Ankara. Collected plants identified by Prof. Dr. Mecit Vural. Samples were stored at +4°C. The method of Özcan and Şenyuva (2006) was used in the extraction for the analysis of free amino acids. For the purpose of total protein analysis, all pollen extractions were obtained from samples which were collected in 2000. The analysis conducted by adding some modifications to the method developed by Evans (1991) and protein analysis was carried out by applying the Lowry method (Lowry *et al.*, 1951).

**Reagents:** Alanin (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), cystine (Cys-Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly),

histidine (His), hydroxyproline (Hyp), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), tryptophan (Trp), valine (Val) standards were supplied by Aldrich (Milwaukee, USA). Formic acid (98%) and acetic acid (glacial) were analytical grade obtained from Merck (Darmstadt, Germany). Ultra pure-water was used through the experiments (Milli-Q system, Milli-pore, Bedford, Ma, USA).

**Instrumentation:** Glass vials with septum screw caps and analytical column Zorbax Bonus-RP, Narrow-Bore RP (100×2.1 mm, 3.5 μm), Zorbax SB Aq (150×4.6 mm, 3.5 μm) and Zorbax Eclipse XDB C 18 (75×4.6 mm, 5 μm) were supplied by Agilent Technologies (Wilmington, DE, USA), Ace 3 C18 (100×2.1 mm, 3 μm) was supplied by ACE-HPLC (Reading, UK), Hichrom Inertsil ODS 3 (250×4.6 mm, 3.5 μm) was purchased from Hichrom (Berkshire, UK), Heidolph Silentcrusher M homogenizer (Donau, Germany) and digital pH meter Mettler ToledoMP220 (Leicester, UK).

The LC/MS analysis for the screening and quantification of 21 free amino acids were performed by Agilent 1100 HPLC system (Waldbronn, Germany) consisting of a binary pump, an autosampler and a temperature controlled column oven, coupled to an Agilent 1100 MS detector equipped with APCI interface. The analytical separation was performed on a Zorbax Bonus -RP, Narrow-Bore (100×2.1 mm, 3.5 μm) using the isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid at a flow rate of 0.2 mL min<sup>-1</sup>. Data acquisition was performed in Selected Ion Monitoring (SIM) mode using the interface parameters. The analytical separation was performed on a Zorbax Bonus-RP, Narrow Bore (100×2.1 mm, 3.5 μm) using the isocratic mixture of 0.01 mM acetic acid in 0.2% aqueous solution of formic acid at a flow rate of 0.2 mL min<sup>-1</sup>. Full scan analyses were performed in the mass range of 50-500 da for the spectral identification of amino acids and sample co-extractives, respectively. The ions of 21 amino acids were monitored for the screening and quantifying of amino acids in samples.

**Sample preparation:** Stock solutions of amino acids 1000 μg mL<sup>-1</sup> were prepared by dissolving 25 mg of each in 25 mL distilled water. Working standards were prepared by diluting the stock solution of amino acids to concentrations of 0.05-5.00 μg mL<sup>-1</sup> with 0.2 mM acetic acid.

Samples were weighed in to a 10 mL glass centrifuge tube with cap. Ten milliliters of 0.2 mM acetic acid was added to the samples. After mixing in a vortex for 2 min,

the mixture was centrifuged at 5000 rpm for 10 min at -5°C. The clear supernatant was quantitatively transferred in to a vial avoiding the top oil layer if present. It was filtered through 0.45 μm nylon syringe filter prior to LC/MS analysis (Desai and Armstrong, 2004; Gu *et al.*, 2007).

**Quality assurance:** Quality assurance measures were employed for amino acids which involved inclusion in each batch of 8 samples, duplicate samples spiked at 5, 10, 50 mg/100 g and reagent blank. Batches of samples were deemed acceptable if spiked samples indicated better than 80% recovery.

## RESULTS AND DISCUSSION

A total of 21 amino acids were found in examined taxa. The amino acid contents of investigated taxa are given in Table 1. The results revealed that the amino acid content ranges from 2.02 to 2.353,61 mg/100 g. Amino acids such as arginine, asparagine, proline, tyrosine, glutamine, histidine, lysine and alanine were present abundantly among all taxa. Studied pollens included limited amounts of glycine and glutamic acid. The other major amino acid present included leu-isoleucine, valine, tryptophan, phenylalanine, serine, cysteine, hydroxyproline, aspartic acid and cystine. Hydroxyproline, aspartic acid, methionine and tryptophan were present in trace amount in *F. angustifolia* which was collected in 2000. Histidine was not present in also *F. angustifolia* in stored pollen. Arginine was present in *P. nigra* subsp. *nigra* var. *caramanica* at most.

The results of total protein analyses showed that the total amounts of protein are remarkably higher in *P. nigra* subsp. *nigra* var. *caramanica* than in the other taxa (Table 2).

Pollen grains of examined taxa showed huge variability in the amount of free amino acid content. Prior studies noted strong relationship between the amount of amino acid composition found in pollens and external conditions of plant such as climatic and nutritional conditions as well as with storage and handling patterns (Mondal *et al.*, 1998b; Singh *et al.*, 1992). Shahali *et al.* (2007) observed that *C. arizonica* pollen protein content might be influenced by environmental conditions. The present study was designed to determine the changing pattern of amino acid composition of same taxa in time. What is remarkable in the obtained results is that there is no clear trend of decrease or increase in the amount of amino acid composition in time in different taxa; on the contrary, free amino acid content of fresh and stored pollen grains shows unique changes in each taxon. For instance pollen of *J. regia* and *P. nigra*, the amount of

Table 1: Content of free amino acids in examined pollen grains (mg/100 g)

Amino acids	<i>P. nigra</i> (2000)	<i>P. nigra</i> (2008)	<i>B. pendula</i> (2000)	<i>B. pendula</i> (2008)	<i>J. regia</i> (2000)	<i>J. regia</i> (2008)	<i>F. angustifolia</i> (2000)	<i>F. angustifolia</i> (2008)
Ala	57.86	108.59	28.19	31.81	135.43	219.32	57.31	270.19
Arg	2.35361	1.40957	88.96	698.67	759.98	396.53	32.78	259.46
Asn	173.55	130.31	86.23	322.43	190.49	58.50	4.40	540.20
Asp	15.09	31.02	13.04	81.46	39.48	25.85	2.31	87.47
Cys	9.25	23.62	9.92	30.67	21.28	12.45	5.07	26.01
Cys-Cys	21.46	35.26	9.04	26.40	24.19	5.88	12.11	61.97
Glu	14.63	29.28	6.41	9.81	15.86	6.76	13.68	35.62
Gln	228.03	165.82	92.96	117.17	171.49	24.67	233.57	314.52
Gly	4.86	10.03	6.14	7.69	10.58	17.18	4.89	3.98
His	206.98	115.40	18.02	355.24	69.92	124.10	0.00	491.07
Hyp	19.89	16.63	5.79	17.31	34.30	69.95	2.02	26.20
Leu	33.34	24.02	25.57	47.07	98.14	27.52	7.52	88.10
Lys	75.94	89.89	38.30	96.75	133.11	64.53	6.67	275.94
Met	16.60	12.60	3.75	7.65	32.54	9.61	2.22	18.04
Phe	38.71	42.46	15.74	30.85	95.92	25.80	5.58	70.59
Pro	673.50	426.01	176.03	833.63	546.53	378.63	8.40	548.47
Ser	22.96	25.05	22.82	23.80	25.96	42.77	7.56	30.67
Thr	13.07	32.48	21.07	10.45	36.81	40.82	8.48	68.79
Tyr	159.66	255.28	38.35	120.52	48.19	66.09	10.75	281.07
Trp	26.97	30.55	15.03	102.44	51.52	14.52	3.15	39.93
Val	34.71	24.86	36.16	37.23	73.18	35.97	11.77	85.45

Table 2: Total protein content of pollen grains (%)

Content of pollen grains (%)	Values
<i>P. nigra</i>	25.75
<i>B. pendula</i>	7.73
<i>J. regia</i>	7.55
<i>F. angustifolia</i>	13.67

arginine, proline and asparagine is much higher in stored pollen grains than fresh pollen grains. These findings are consistent with those of Kim *et al.* (1987), who found that arginine and proline are highest concentrations in black spruce. Earlier study has reported that proline was abundantly present in the investigated nine Asteraceae species (Mondal *et al.*, 1998b). While, the amount of arginine was recorded to be the highest in *P. nigra* subsp. *nigra* var. *caramanica* in stored pollens, alanine was recorded at low levels in *P. nigra* subsp. *nigra* var. *caramanica*, *B. pendula*, *J. regia* and *F. angustifolia* in stored pollen. *B. pendula* and *F. angustifolia* samples that have been collected at 2000 had a smaller content of arginine and asparagine. Histidine was present in *F. angustifolia* collected in 2008 whereas it is recorded to be in lower levels *B. pendula* and *J. regia* pollen grains which were collected in 2000 and completely absent in pollens collected in 2000. The content of tyrosine was high level in fresh pollen in all examined taxa (Table 1). Lysine, glutamic acid and aspartic acid have important roles in maintaining the allergenic activity of the allergen molecule (King *et al.*, 1974; Karmakar and Chatterjee, 1992). Lysine levels were much higher in *J. regia* which was collected in 2000 and *F. angustifolia* which was collected in 2008 than the remaining taxa. The content of aspartic acid and glutamic acid were high in *F. angustifolia* which was collected in 2008. Karmakar and Chatterjee (1992) isolated two highly active allergens ( $C_n$  II and  $C_n$  VII) from *Cocos*

*nucifera* pollen extract. The authors have reported that  $C_n$  II and  $C_n$  VII contains high amount of glycine, alanine, serine, valine and low amount of histidine, methionine, isoleucine, lysine, arginine, proline, tyrosine, phenylalanine, serine and tryptophan. But cystine were high amount in  $C_n$  VII and a little amount in  $C_n$  II.

Amino acids can directly or indirectly influence the physiological activities of the plant. Proline, lysine, methionine and glutamic acid, glutamine, aspartic acid and asparagine are necessary for fertility of pollen and pollination (Kim *et al.*, 1987; Mondal *et al.*, 1998b; Jianjun *et al.*, 1995; Rashed *et al.*, 1995; Krogard and Anderson, 2006). Leu-isoleucine, valine, tryptophan, phenylalanine, serine, cysteine, hydroxyproline, aspartic acid and cystine were other major amino acids. Hydroxyproline, aspartic acid, methionine and tryptophan were present in trace amount in *F. angustifolia* which was collected in 2000.

The results of protein analysis showed that total amounts of protein were much higher in *P. nigra* subsp. *nigra* var. *caramanica* than other examined taxa. *B. pendula* and *J. regia* pollen grains had similar values (Table 2). Total amounts of protein of arborescent plants done with Lowry methods were reported 13.45% in *Pinus radiata* D. Don (stone pine), 11.36% in *P. sabiniana* Dougl. (gray pine), 7.6% in *P. canariensis* C. Smith (Canary Island pine), 24.27% in *Acer negundo* L. (box elder), 46.53% in *A. platanoides* L. (Japanese maple), 37.08% in *Salix babylonica* L. (babylon willow), 10.47% in *Populus thevestina* Dode (theves poplar)(10.47%) (Aytuğ, 1967; Vik *et al.*, 1987; Pehlivan *et al.*, 2003). In another study, Mandal *et al.* (1993) reported that *Spathodea campanulata* Beauv. has 12.74% protein and *Madhuca indica* Gmel. 11.58%.

The results indicate that alanine, arginine, asparagine, glutamine, histidine, lycine, proline and tyrosine constitute major free amino acids in all examined taxa. The results of our study conform with other investigations on the free amino acid content in pollen grains (Nátrova, 1968; Krogaard and Anderson, 2006). The differences of the contents of amino acids between fresh and stored pollen grains could be due to variations depend on environmental conditions, climatic factors, soil composition and stored conditions. Due to the fact that the amino acid content of pollen grains can vary in time, pollen extracts used in the treatment of allergic disorders should be prepared from fresh pollens and also pollens must be obtained from vegetation areas close to the locations where the patients.

#### ACKNOWLEDGMENTS

The authors thank Prof. Dr. Mecit Vural for identification the plant samples. The authors offer their special thanks to Evren Cabi and Umut Toprak for their efforts in editing the manuscript.

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