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Electrophoretic Investigation of Isoenzymes and Soluble Proteins Between Two Varieties of *Vicia sativa* L. Seeds

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Abstract: Improving an identification method was purposed using electrophoretic profiles of seed proteins and four isoenzymes of Karaelci and Sarielci varieties of *Vicia sativa* L. In addition five different extraction buffers were tested for seed proteins. Also seed proteins and four isoenzymes polymorphism between two varieties were investigated. Electrophoresis was carried out with polyacrylamide gel electrophoresis (PAGE). Catechol oxidase and α-glycerophosphate dehydrogenase isoenzymes exhibited identical patterns but esterase and superoxide dismutase isoenzymes showed different patterns. These two isoenzymes, which showed polymorphism, can use as marker suchlike studies with these varieties. Seed proteins band patterns among five buffers were different. One of the buffers revealed maximum band numbers. This buffer was the best seperator buffer. Isoenzymes and seed proteins band patterns which find out can be useful for identification.

Key words: Soluble proteins, isoenzyme, PAGE, *Vicia sativa*

INTRODUCTION

Vicia sativa (common vetch), which is one of the mostly cultivated plants among annual legumes (*Leguminosae*), is an important feed plant due to the high amount of protein content in its seeds.

Proteins, besides being food sources for humans and animals, also provide structural information regarding the identification of an organism and contribute to the determination of evolutionary history of the organism. Due to this reason, electrophoretic analyses contribute us in; revealing the purity of increasing number of varieties, identification of varieties, assisting biosystematic analyses, marking the phylogenetic relationships among species and supporting the evolution by increasing the knowledge on genetical diversity (Sammour, 1991).

The goal of this study is to improve an identification method and to determine the differences and similarities for the summer form of *Vicia sativa* L. with black seeds (Karaelci common vetch L-147) and the winter form with yellow seeds (Sarielci common vetch D_3-240) by the determination of electrophoretic band patterns of soluble proteins in the seeds via SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) and those of esterase (EST), superoxide dismutase (SOD), cathechol oxidase (CO) and α-glycerophosphate dehydrogenase (α-GPDH) isoenzymes via Native-PAGE method which would consequently provide supportive knowledge to be used in molecular biology and taxonomy studies. Moreover, in these seeds, different extraction buffers have been tried in SDS-PAGE method in total protein studies for determining the best separator buffer.

MATERIALS AND METHODS

This study was conducted in Ankara University, Faculty of Science, Department of Biology laboratories at from February to June 2005.

Plant materials: Two variety (Karaelci and Sarielci) of *Vicia sativa* L. seeds were used. Seeds were obtained from Ankara University, Faculty of Agriculture, Department of Field Crops.

Extraction for total proteins and SDS-PAGE: Five extraction buffers (buffer I+II, buffer II, buffer IIa, buffer IIb, buffer IIc) were used prior to the SDS-PAGE process which was used for the electrophoresis of the total proteins in the seeds.

Buffer I+II: One of these 5 buffers (Buffer I), which is the suggested extraction buffer by Arulsekar and Parfitt (1986), is actually used for isoenzyme extraction and also was used in SDS-PAGE process for the evaluation of total protein extraction performance. Therefore supernatant, which obtained then extraction with buffer I, was mixed with buffer II (1:1) (v:v).

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Buffer I {containing: 0.05 M Tris base 6.5 g L^{-1}; 0.007 M citric acid (monohydrate) 1.5 g L^{-1}; 0.1% cysteine hydrochloride 1 g L^{-1}; 0.1% ascorbic acid (Na salt or free acid) 1 g L^{-1}; 1.0% polyethylene glycol (M_r 3500) 10.0 g L^{-1}; 1 mM 2-mercaptoethanol 0.08 mL L^{-1}. The final pH 8.0}.

Buffer II {containing: 0.0625 M Tris-HCl (pH 6.8); 2% sodium dodecyl sulphate; 10% glycerolol; 5% 2-mercaptoethanol; 0.001% brom phenol blue} (Laemmli, 1970).

Buffer IIa {containing: 0.0625 M Tris-HCl (pH 6.8); 10% glycerolol; 0.001% brom phenol blue}

Buffer IIb {containing: 0.0625 M Tris-HCl (pH 6.8); 2% sodium dodecyl sulphate; 10% glycerolol; 0.001% brom phenol blue}

Buffer IIc {containing: 0.0625 M Tris-HCl (pH 6.8); 10% glycerolol; 5% 2-mercaptoethanol; 0.001% brom phenol blue}

Buffers IIa, IIb, IIc were the modified forms of buffer II. The aims of these modifications were realizing the extractions either by breaking off or unbreaking the dissulphide (S-S) bonds of proteins and examining the changes in band patterns. Buffer IIa was used for the extraction without breaking off dissulfide bonds (non-reducing conditions) and SDS and 2- mercaptoethanol (2-ME) were not added to the content. For the extractions by breaking off the dissulphide bonds (reducing conditions), buffers II, IIb, IIc were used. The SDS and 2-ME used for developing the reducing conditions were existent in the content of buffer II. While only SDS was added to the content of buffer IIb, only 2-ME was added to the content of buffer IIc.

The seeds of Karaelci and Sarielci common vetch were pounded in the mortar till they were powdered finely. 0.5 g seed flour and 1500 μL from each of the mentioned different buffers were mixed in homogenizator tubes. Homogenization was carried out in the electrical homogenizator for 1 min. Homogenates in the tubes were transferred into eppendorf tubes and centrifuged at room temperature at 18000 rpm for 20 min. Supernatants were transferred into the new eppendorf tubes and pellets were discarded. The supernatant obtained from the tubes prepared with buffer I was mixed with buffer II at 1:1(v:v) ratio. The reason for that was to increase the density with the glycerol in buffer II and to monitor the electrophoresis process with the tracking dye, brom phenol blue. Moreover, buffer II constituted the reducing conditions as well due to its SDS and 2-ME content. The samples were stored at -20°C until electrophoresis process. Prior to electrophoresis, they were kept at 100°C boiling water for 5 min for denaturation and after recenrifuged at 18000 rpm for 5 min.

SDS-polyacrylamide denature gels were prepared in a concentration of 10% resolving gel and 4% stacking gel as suggested by Sambrook *et al.* (1989). Electrophoresis was carried out according to Laemmli (1970). The protein amount to be loaded to the gel was determined semiquantitatively according to Esen (1978).

Extraction for isoenzymes and PAGE: Prior to isoenzyme extraction, having been kept in distile water for seven days, seeds were taken to imbibition process. Throughout seven days, each day, 10 imbibed yellow seeds and 10 black seeds were extracted as suggested by Arulsekar and Parfitt (1986).

Nondenaturing polyacrylamide gels were prepared in a concentration of 7.5% resolving gel and 4% stacking gel as suggested by Sambrook *et al.* (1989). Electrophoresis was carried out according to Laemmli (1970). The extracts were thawed and mixed with 10% sucrose which contain bromophenol blue at 1:1 ratio (Prabhakaran and Kamble, 1993). Fifty microliter from each sample was loaded to the gels. After electrophoresis, each gel was stained with different stains for different enzymes: The methods suggested by Scandalios (1969) and Turkben *et al.* (2002) were used for Esterase (EST) and cathechol oxidase (CO) enzymes, respectively. The method suggested by Harris and Hopkinson (1976) was used for alpha-glycerophosphate dehydrogenase (α-GPDH) and superoxide dismutase (SOD) enzyme. Acromatic bands were evaluated as SOD and dark bands were evaluated as α-GPDH.

RESULTS

Total protein band patterns: As far as the electrophoretic band numbers in the SDS-PAGE of seed proteins of Sarielci and Karaelci common vetch are analyzed, it has been seen that the extracts of the same sample prepared in different extraction solutions have displayed different band numbers. For example, while the extracts of Sarielci seeds prepared in Buffer I+II displayed a band number of 24, the band number displayed by the extracts prepared in Buffer II was found to be 21. Furthermore, band density was also observed to vary according to the buffers used (Fig. 1).

Intervarieties variations: When the electrophoretic band patterns of Sarielci and Karaelci varieties are compared among themselves for each buffer, the results obtained in terms of band number and mobilities are summarized in Table 1.

Table 1: SDS-PAGE band numbers of extracts of Sarielci ve Karaelci seeds in different buffers (MW: molecular weight, kD: kiloDalton)

	Buffer I+II		Buffer II		Buffer IIa		Buffer IIb		Buffer IIc	
MW (kD)	Sarielci	Karaelci	Sarielci	Karaelci	Sarielci	Karaelci	Sarielci	Karaelci	Sarielci	Karaelci
>205	0	1	0	0	0	1	1	0	0	0
205-116	1	1	2	1	1	1	4	2	1	1
116-97.4	1	0	1	0	0	0	0	0	0	0
97.4-66	6	5	6	6	2	1	4	5	3	3
66-45	4	4	5	5	4	2	4	4	4	4
45-29	5	7	4	5	5	9	5	6	6	4
<29	7	6	3	3	4	4	3	4	4	4
Total	24	24	21	20	16	18	21	21	18	16

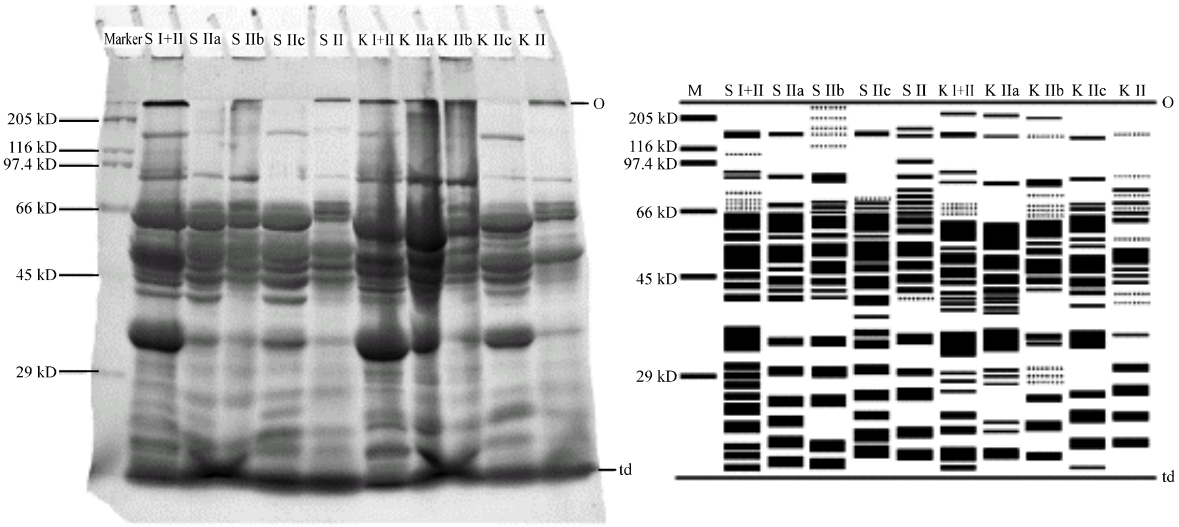

Fig. 1: SDS-PAGE band patterns of Sarielci (S) and Karaelci (K) seed storage proteins (I+II, IIa, IIb, IIc, II: extraction buffers, M: marker, kD: kiloDalton, O: origin, td: tracking dye)

Interbuffers variations

Sarielci: Differentiations were noted also among the band patterns of Buffers I+II, II, IIa, IIb, IIc when compared. The total band numbers were found to be 24 in Buffer I+II; 21 in Buffer II; 16 in Buffer IIa; 21 in Buffer IIb and 18 in Buffer IIc (Table 1).

Karaelci: Total band number was found to be 24 in Buffer I+II; 20 in Buffer II, 18 in Buffer IIa, 21 in Buffer IIb and 16 in Buffer IIc (Table 1).

Isoenzyme band patterns

Esterase (EST)

Karaelci: Three EST enzyme loci were observed. Loci were named as $EST1_k$, $EST2_k$ and $EST3_k$ All the loci were identified as homozigous. In the first ($EST1_k$) and second loci ($EST2_k$), the densities of the bands on the 1st day of imbibition were found to be low. In the third locus ($EST3_k$), an EST band was determined on the 1st, 2nd, 3rd, 4th and 5th days of imbibition which, however, was non-existent on the 6th and 7th days (Fig. 2).

Sarielci: Three EST enzyme loci were observed in this variety as well. Loci were again named as $EST1_s$, $EST2_s$ and $EST3_s$ It was found that first locus ($EST1_s$) displayed the same mobility as the second locus ($EST2_k$) of Karaelci did. In all of the three loci, Sarielci was determined as homozigous in terms of EST. The bands on the 1st and 2nd days of imbibition appeared as unclear in the third locus ($EST3_s$) (Fig. 2).

Cathechol Oxidase (CO): Single CO enzyme locus was noted in Karaelci and Sarielci. In both varieties, enzymes appeared as homozigous and were fixed for the same allel (Fig. 3).

Superoxide dismutase (SOD) and Alpha-glycerophosphate dehydrogenase (α-GPDH): Alpha-glycerophosphate dehydrogenase activity staining was applied for SOD. Concerning α-GPDH, single locus was observed in both varieties which were fixed for the same allel. Gel was then exposed to light. After the darkening of

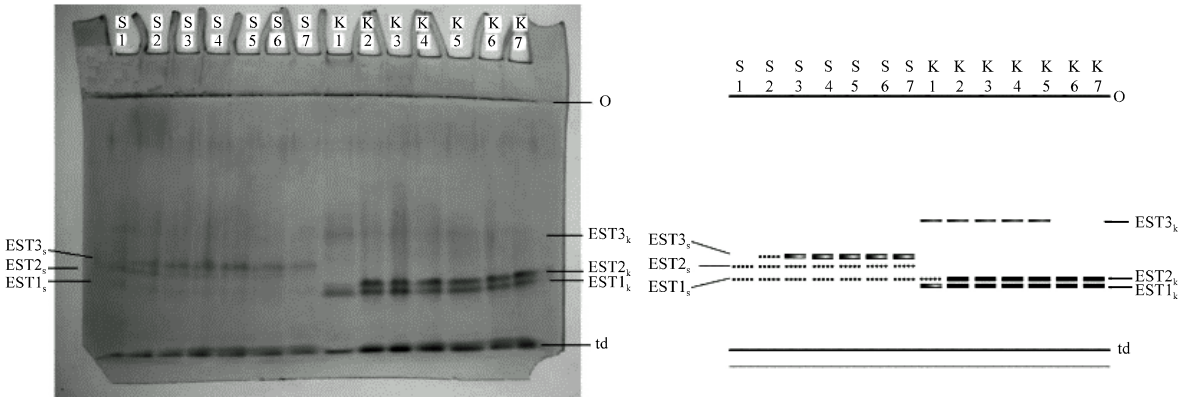

Fig. 2: PAGE band patterns of Sarielci's (S) and Karaelci's (K) EST isoenzymes on the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th days of imbibition (O: Origin, td: tracking dye)

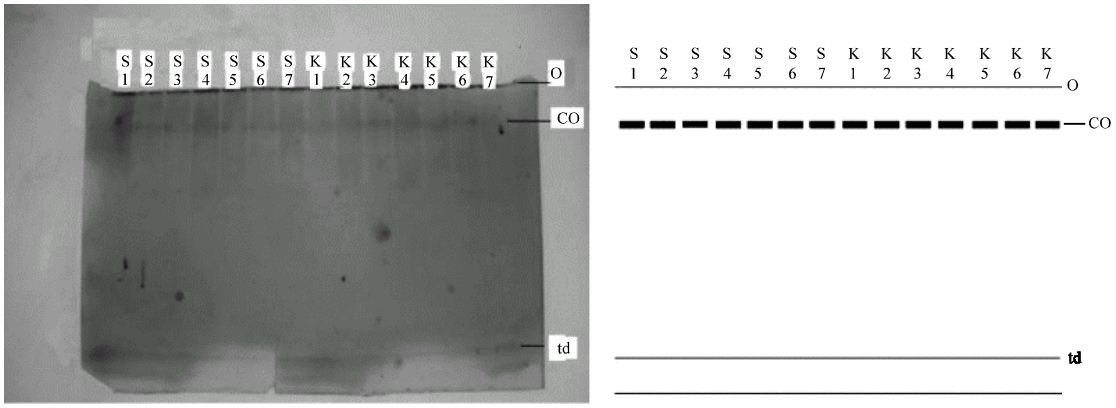

Fig. 3: PAGE band patterns of Sarielci's (S) and Karaelci's (K) CO isoenzymes on the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th days of imbibition (O: Origin, td: tracking dye)

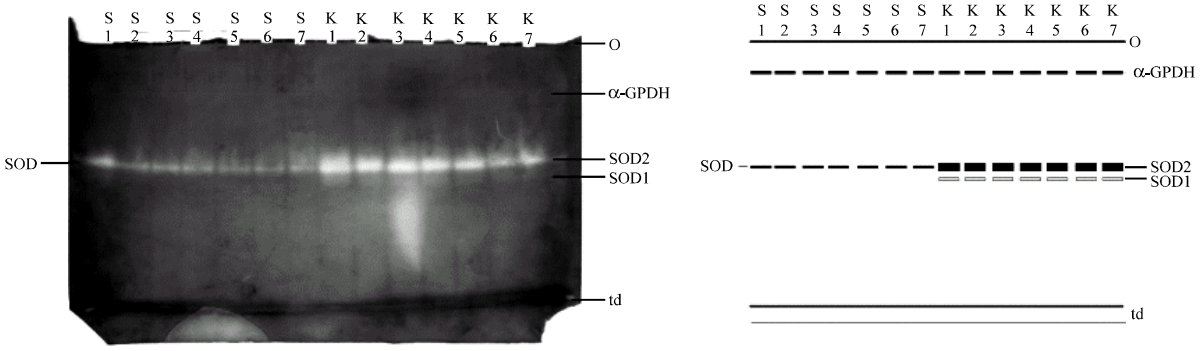

Fig. 4: PAGE band patterns of Sarielci's (S) and Karaelci's (K) SOD and α-GPDH isoenzymes on the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th days of imbibition (O: Origin, td: tracking dye)

the gel background, SOD bands appeared as acromatic zones. Two loci in Karaelci and one locus in Sarielci were determined. Karaelci loci were named as SOD1 and SOD2. While SOD1 displayed a weak activity zone, stronger activation was observed in SOD2. Sarielci SOD locus allels had the same mobility levels as Karaelci SOD2 locus allels and demonstrated medium level of activation (Fig. 4).

DISCUSSION

In Haider and El-Shansoury (2000), study the seed proteins of 6 subspecies of *Vicia sativa* L. were examined with SDS-PAGE and parallel to our findings, differences were determined among the subspecies alongside the similarities. They have stated that the similarities among the subspecies studied were high at the kD ranges 97-65 and 32-29. The extraction buffer used by those researchers is the same with the Buffer II used in this study, except the use of sucrose instead of glycerol. The highest similarities in the SDS-PAGE process with Buffer II were observed at the kD ranges 97, 4-66 and 32-29 also in this study. It has been seen that these findings in both studies are almost the same and compatible with each other. Based on these findings, it is possible to conclude that the MW's of similar seed proteins of *Vicia sativa* L. subspecies and varieties are at the kD ranges 97-66 and 32-29.

Valizadeh (2001), by using SDS-PAGE, examined the electrophoretic band patterns of salt-soluble seed proteins of 11 *Leguminosae* species cultivated in Iran. Valizadeh (2001) found out that the band patterns of proteins were completely the same among different accessions and based on this fact; he stated that SDS-PAGE could determine interspecies seed protein polymorphisms, but not those in varieties and populations. Contrary to Valizadeh (2001), in this study, the band patterns of the seed proteins of the two different varieties varied in all of the buffers used. Therefore, it is considered that identification of Sarielci and Karaelci varieties could be done by polymorphism of the seed proteins.

In a SDS-PAGE study conducted by Sammour (1999) in *Linum usitatissimum* L. seeds, it has been noted that mainly 6 bands of 55-39 kD appeared in non-reducting conditions. However, after the reducing process with 2-ME, the band patterns changed remarkably and, the 6 bands disappeared and bands around 40 and 20 kD appeared instead. In this study, the bands at high MW did not disappear as remarkably as they did in Sammour (1999), study. However, band patterns observed in the extracts of different buffers used for reducing and non-reducing conditions displayed variations. In the study, Buffer I+II was determined as the mixture displaying the highest band number with a total of 24 bands for both of the varieties. These indicate that Buffer I+II constituting the reducing conditions breaks the dissulphide bonds better. Moreover the band densities are clearer in the extracts prepared with Buffer I+II when compared to those prepared by the other buffers. This shows that seed proteins are much better solved in this solution. It has been thought that this difference between this study and Sammour (1999), study might have stemmed from the differences in the extraction buffers used and differences in extraction processes.

Dirk *et al.* (1995) have stated that the number and activities of isoenzymes have increased in the imbibed seeds. Indeed, more activities were achieved as well in this study in the electrophoresis processes carried out with the imbibed seeds.

In Haider and El-Shansoury (2000), study it has been noted that; the esterase band patterns of 6 *Vicia sativa* L. subspecies have shown striking difference relative to the number of loci and allels among the subspecies. The number of esterase bands varied between 2-4 and except *V. sativa* subspecies *nigra,* the same EST band pattern was observed in the different accessions. It has been stated that in *V. sativa* subsp. *sativa*, esterase enzyme appeared as dimer with single allel in each of the two of homozigous loci. When the zymogram was examined, two bands were determined in both of the samples of this subspecies. As they identified the two bands as homozigous and single allel, it could not be clarified on which grounds this enzyme was considered as dimer as it is impossible to comment on the quaterner structure of the enzyme unless heterozygous individuals are observed in the same population. When the esterase band patterns of Karaelci and Sarielci varieties were examined in this study, differences were observed. While there were 3 loci in both varieties, one of them was same and the others were different. All loci appeared as homozigous in both varieties. Due to this fact, no comment could be made pertaining to the quaterner structure of the enzyme.

The low band density in the first ($EST1_k$) and second loci ($EST2_k$) on the 1st day of imbibition in Karaelci indicates that probably the enzyme did not yet acquire its activity on the 1st day of imbibition. The determination of an EST band pattern in the third locus ($EST3_k$) appearing on the 1st, 2nd, 3rd, 4th and 5th days of imbibition but non-existent on the 6th and 7th days might indicate the loss of activity in the enzyme as of the 5th day. The similar situation seen in the third locus ($EST3_s$) of Sarielci as the appearance of the weak bands on the 1st and 2nd days of imbibition might indicate that this isoenzyme was not active either on the first two days.

No study could be found pertaining to CO isoenzyme in *Vicia sativa* L. However, the CO variations were evaluated among *Vicia canescens* populations. As material, leaves and their stems were used rather than

plant seeds and it has been noted that CO enzyme has shown fairly polymorphic characteristics. This range of variation of the enzyme among populations were thought to be explained probably by the variation of CO according to plant tissues. In this study, CO enzyme was found to display identical band patterns in Karaelci and Sarielci. If the study was carried out with different populations or different plant tissues, perhaps a highly polymorphic CO enzyme could have been determined similarly.

No electrophoretic study could be found regarding SOD enzyme carried out with Karaelci and Sarielci varieties. Jaaska (1997) examined with PAGE the isoenzyme composition of 10 different enzymes of 21 *Vicia* species. He stated that among 4 *Vicia sativa* subspecies, one has displayed a different pattern than the other 3 subspecies. He expressed that SOD isoenzymes appeared as SOD-A (chloroplastic), SOD-B (cytosolic), SOD-C (mitochondrial) and when dithiothreitol and diethyldithiocarbamate were added to the extraction buffer as sulphydryl protector, SOD-A and SOD-B containing Cu:Zn were inhibited while SOD-C containing Mn was not affected. It was concluded that, of the heterozygous individuals appearing in the species except *V. sativa*, SOD-A and SOD-B were dimeric and SOD-C was tetrameric enzymes. Differences were determined between the two varieties also in this study. Actually, one of the loci was identical, but another locus was observed in Karaelci that was non-existent in Sarielci. As no inhibition procedures were conducted in this study, it was not possible to identify the types of the SOD loci. Therefore, 2 and 1 loci were determined in Karaelci and Sarielci, respectively. No comment could be made about the quaterner structure due to the homozigous loci in both varieties.

As a conclusion the electrophoretic analyses of seed proteins and four enzymes in Karaelci and Sarielci varieties indicated these band patterns which find out can be useful for identification, but joint analysis of more enzymes will increase the correctness of the tests. Though no variations in CO and α-GPDH enzymes were determined between the two varieties, EST and SOD were found to vary. These two isoenzymes, which showed polymorphism, can use as marker suchlike studies with these varieties. Seed proteins band patterns among five buffers were different. Buffer I+II revealed maximum band numbers. This buffer was the best seperator buffer.

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