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Research Article

ALLELE FREQUENCY OF GLUTENIN SUBUNITS AND GLU-1 QUALITY SCORES IN SOME TURKISH BREAD WHEAT LANDRACES

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Abstract: There are eight centers of origin for cultivated plants and Turkey is located in the interception of two of these centers, the Near East and the Mediterranean. Therefore, Turkey is known to be the gene center for diversification and dispersion of such main cereal crops such as wheat, barley, rye and oat. This study was performed to determine glutenin gene allele frequencies and *Glu-1* quality scores of 116 local wheat landraces of Turkish bread wheat. SDS-PAGE and PCR were used to identify glutenin gene alleles. The results showed that the studied Turkish local wheat landraces contained a total of 19 different subunits (3 subunits in *Glu-A1*, 11 in *Glu-B1* and 5 in *Glu-D1*) with 50 different combinations. The highest and the lowest allelic combinations were determined in East Anatolia and the Aegean regions, respectively. *Glu-A1*c (65.11%), *Glu-B1*b (53.60%) and *Glu-D1*a (58.30%) were the most frequent alleles. The *Glu-1* quality score was found to be 6.07 for the studied genotypes. Among the regions, the highest (7.18) and the lowest (4.80) mean *Glu-1* scores were detected in Marmara and Southeastern Anatolia regions, respectively. 4 accessions (TR32846-6, TR36948-1, TR45105 and TR63536) were reported to have the highest *Glu-1* quality score as 10. 6 genotypes (TR45398-4, TR48025-3, TR33264-6, TR393-5, TR52021-3 and TR45094) had the quality score of 9. Including more new landraces may contribute to discover new *Glu-1* alleles.

Özet: Kültür bitkileri için sekiz orijin merkezi vardır ve Türkiye, bu merkezlerden ikisinin, Yakın Doğu ve Akdeniz'in, kesiştiği yerdedir. Türkiye bu nedenle buğday, arpa, çavdar ve yulaf gibi ana tahıl bitkilerinin çeşitlendiği ve dağıldığı gen merkezi olarak bilinir. Bu çalışmanın amacı, Türk ekmeklik buğdaylarından 116 yerel buğday ırkının glüten allel sıklığı ve *Glu-1* kalite skorunu belirlemektir. SDS-PAGE ve PCR, glüten allellerini tanımlamak için kullanılmıştır. İstatistiksel analizler için POPGENE 1.31 yazılımı kullanılmıştır. İncelenen Türk yerel buğday ırkları toplam 19 farklı alt birim (*Glu-A1*'de 3 alt birim, *Glu-B1*'de 11 ve *Glu-D1*'de 5 alt birim) ve 50 farklı kombinasyon içermektedir. En yüksek ve en düşük allel kombinasyonları sırasıyla Doğu Anadolu ve Ege bölgelerinde belirlenmiştir. *Glu-A1c* (% 65,11), *Glu-B1b* (% 53,60) ve *Glu-D1a* (% 58,30) en sık görülen alleller olarak tespit edilmiştir. *Glu-1* kalite skoru, Türkiye genelinde incelenen genotipler için 6,07 olarak bulunmuştur. Bölgeler arasında ortalama en yüksek (7,18) ve en düşük (4,80) *Glu-1* skorları sırasıyla Marmara ve Güneydoğu Anadolu bölgelerinde tespit edilmiştir. Çalışılan 116 aksesyondan 4 tanesi (TR32846-6, TR36948-1, TR45105 ve TR63536) kalite skoru 10 olup en yüksek Glu-*1* kalite skoruna sahipken 6 tanesinin ise (TR45398-4, TR48025-3, TR33264-6, TR393-5, TR52021-3 ve TR45094) kalite skoru 9 olarak hesaplanmıştır. Daha fazla yerel çeşitlerin çalışmalara eklenmesi yeni *Glu-1* allelerinin keşfedilmesine katkıda bulunabilir.

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Introduction

Vavilov (1951) identified eight centers with gene center status that have long been used for agriculture and Turkey is located in the interception of two of these centers (Near East and the Mediterranean), making the country one of the richest in terms of plant biodiversity. Turkey is also known to be one of the gene centers for diversification and dispersion of main cereal crops such as wheat, barley, rye, and oat. This special future, in addition to being the first place where wheat was cultivated, makes the country for several wild species and landraces of wheat to be available. For instance, the first cultivated forms of diploid and tetraploid wheat originated from Karacadağ (Diyarbakır) in the Southeastern region of Turkey.

These first diploid (AA) and tetraploid genomes (AABB) and their phylogenetic analysis indicate that they

had originated in Southeastern Turkey (Dubcovsky & Dvorak 2007). Cultivation shifted from here to near east about 9.000 years ago and the hexaploid wheat appeared for the first time (Feldman 2001). Cereals occupy 55% of total agricultural and 57% of cultivated areas (21.4 million hectares) in Turkey (Grain sector report 2013). Among the cereals in Turkey, wheat is the pioneering crop with 67% share and the country supplies 3% of world wheat production every year. However, because of quality-related issues, it is still a wheat importing country (TMO 2017). Among the regions, the leading wheat producing region is Central Anatolia (32.4%), followed by Marmara (17.1%), Southeastern Anatolia (13.3%), the Mediterranean (12.8%), Black Sea (10.1%), Aegean (7.4%) and Eastern Anatolia (6.9%) regions.

The quantity and composition of high molecular weight gluten subunits (HMW-GS) are important factors in determining wheat baking properties. Localization of HMW subunit genes on long arms of homologous group 1 was reported by Orth & Bushuk (1974) and Bietzh *et al*. (1975). Each locus contains two linked genes called x and y type which are distinguished by their characteristics and molecular weights (Payne *et al.* 1981). However, as some of those genes are silent, the common wheat possesses 3 to 5 HMW subunits encoded at the Glu-1 loci on the long arms of group 1 chromosomes (1A, 1B, and 1D). The contribution of D genome, followed by B genome considered to have a significant influence on good baking quality (Uthayakumaran *et al.* 2002). Especially, two subunits are expressed always by *Glu-D1*, one or two by *Glu-B1*, one or none (null allele) is expressed by *Glu-A1* loci. If one subunit is expressed by *Glu-A1* or *Glu-B1*, this is always considered as x-type. Rheological properties of the gluten complex are related to the presence or absence of specific subunits of these proteins. The presence of certain HMW subunits is positively correlated with good bread-making quality to determine gluten elasticity (Nakamura 2000, Shewry *et al.* 2003). The relationships between HMW subunits and dough elasticity were determined 40 years ago (Payne *et al.* 1979). Significant differences were found among protein components of wheat grain depending on cultivars, environments and their interactions (Horvat *et al.* 2015, Tok *et al.* 2011). Allelic variations in each *Glu-1* loci were reported in bread wheat genotypes (Lawrence & Shepherd 1980, Payne & Lawrence 1983) and an enumeration system was developed to define different allelic subunits. The definition of HMW subunits coded by *Glu-D1* and *Glu-A1* was described by Payne *et al.* (1983) and Lafiandra *et al.* (1997).

Subunits of gluten proteins can be identified by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. Considering the fact that chains of gluten polymers are stabilized by disulfide bonds, using reducing agents such as DTT and mercaptoethanol in SDS-PAGE makes the identification of gluten subunits easier.

Although Turkey comes third in the production of wheat in the world, quality problems still persist. This study was performed to determine the composition and occurrence frequency of HMW glutenin subunits and the potential of the end-use quality in Turkish wheat landraces to be used in future breeding programs. Analysis of broad collections of landraces enabled the definition of rare alleles on *Glu-1* loci.

Table 1. The wheat specimens studied with their respective distribution regions. All specimens were given using their gen bank accession numbers.

Regions	Wheats (with gen bank accession numbers)	Total Number
Central Anatolia	TR45324-2, TR48061-4, TR53299-1, TR53343-5, TR55002-3, TR46889-2, TR45094, TR47944, TR63316-4, TR35409-1, TR52021-6, TR52021-3, TR35408-4, TR35147-4, TR45303-4, TR45306-2, TR45306-5, TR45308-2, TR53862-5, TR55153-4, TR55164-1, TR55180-1, TR57999-2, TR57999-6, TR32034, TR63536, TR63538	27
Aegean	TR52860-3, TR52860-6, TR52865-1, TR55127-6, TR55140-1, TR55144-1, TR55174-1, TR55201-4, TR52784, TR52873, TR56099	11
Marmara	TR33264-6, TR51937-1, TR51937-2, TR33500-4, TR33500-6, TR38316-4, TR52645, TR52669, TR51937, TR44365, TR45080, TR26746	12
Mediterranean	TR46804, TR52824, TR55316, TR55110, TR37492-1, TR37492-2, TR37492-4, TR32009-5, 393-5, TR26233, TR62808	11
Black Sea	TR14861-5, TR44487-4, TR46873-4, TR44365-4, TR44365-5, TR44984-5, TR32125-3, TR37234-1, TR45105, TR36948-1, TR46861-3, TR44388-6, TR44433-5, TR48373-1, TR54988-1, TR54989-4, TR37383	17
Eastern Anatolia	TR32650-5, TR32668-2, TR32780-2, TR32846-6, TR45370-2, TR48034-6, TR32231-5, TR32231-6, TR45420-5, TR45422-2, TR32273-4, TR39676-4, TR45402-3, TR45398-4, TR47993-1, TR32761-6, TR32881-2, TR39660-1, TR45105-6, TR48025-3, TR47961, TR48050, TR31894-3, TR31894-6, TR32014-5, TR32218, TR63329, TR63322	28
Southeastern Anatolia	TR32218-1, TR32218-5, TR32218-6, TR50443, TR46810-5, TR46822-2, TR38888-6, TR50455-4, TR49018-1, TR31678	10

Table 2. The sequence data for the primers used and expected fragment sizes.

	Alleles	Primer sequences	Expected fragment sizes (bp)	References
P1	Dx2, Dx5	F: GCCTAGCAACCTTCACAATC R: GAAACCTGCTGCGGACAAG	450	Ahmad (2000)
P2	Dy10, Dy12	F: GTTGGCCGGTCGGCTGCCATG R: TGGAGAAGTTGGATAGTACC	576 612	Ahmad (2000)
P3	By18*,By20*,By8, By8*,By9,By16	F:GCAGTACCCAGCTTCTCAA R:CCTTGTCTTGTTTGTTGCC	290-400	Salmanowicz&Dylewicz (2007)
P4	*Axnull*	F: ACGTTCCCCTACAGGTACTA R:TATCACTGGCTAGCCGACAA	920	Salmanowicz&Dylewicz (2007)
P5	*Ax1 + Ax2**	F:CCATCGAAATGGCTAAGCGG R:GTCCAGAAGTTGGGAAGTGC	1500	Salmanowicz&Dylewicz (2007)
P6	*Ax2**	F:CCGATTTTGTTCTTCTCACAC R:CACCAAGCGAGCTGCAGAT	2652	Salmanowicz & Dylewicz (2007)
P7	By20*,By8,By8* By18*, By9	F:TTCTCTGCATCAGTCAGGA R:AGAGAAGCTGTGTAATGCC	750, 710, 660	Salmanowicz & Dylewicz (2007)
P8	*Bx7*	F:ATGGCTAAGCGCCTGGTCCT R:TGCCTGGTCGACAATGCGTCGCTG	2373	Ahmad (2000)
P9	By8	F:TTAGCGCTAAGTGCCGTCT R:TTGTCCTATTTGCTGCCCTT	527	Salmanowicz & Dylewicz (2007)
P10	*Bx14* and *Bx17* cauBx752	F:AGGGGCAGGGAAGAAACACT R:CCAGGCAACACAAATCCATG	642 and 534	Xu *et al.* (2008)
P11	*Bx14* and *Bx17* CauBx642	F:GGGCAATCGGGGTACTTCC R:CCCTTGTCTTGGCTGTTGTC	642 and 534	Xu *et al.* (2008)
P12	Ax2*	F: ATGACTAAGCGGTTGGTTCTT R: ACCTTGCTCCCCTTGTCTTT	1400	Ma *et al.* (2003)
P13	Bx	F: CGCAACAGCCAGGACAATT R: AGAGTTCTATCACTGCCTGGT	650-750	Ma *et al.* (2003)
P14	Bx6, Bx7, Bx7*	F:CAAGGGCAACCAGGGTAC R:AGAGTTCTATCACTGCCTGGT	(850-920), (420-640), (180-280)	Salmanowicz & Dylewicz (2007)
P15	GluD1y10	F:5'-CAACCAATCTCCACAATC-3' R:5'-CTGCAGAGAGTTCTATCA-3'	2210	De Bustos & Jouve (2003)
P16	GluA1-1, 2* Co-dominant Ax2*	F:5'- AAGACAAGGGGAGCAAGGT-3' R:5'- GTGCTCCGCGCTAACATG-3'	1090 1063	Radovanovic & Cloutier (2003)
P17	GluB1 7*, H7 Dominant Bx7	F:5'- CAACAACTTGTGGGGGCCTT-3' R:5'-GCGCTTAGCCATCTCAGTGAAC-3'	1116	Radovanovic & Cloutier (2003)
P18	Co-dominant Bx7	F:5'-ACCTCAGCATGCAAACATG-3' R:5'- GCGCTTAGCCATCTCAGTGAAC-3'	530, 1259, 1302, 3200	Radovanovic & Cloutier (2003)
P19	GluD1- 2, 5 Dx5 Dominant	F:5'-CGTCCCTATAAAAGCCTAGCC-3' R:5'-GGCTAATGTCTCGGAGCTGT-3'	272	Radovanovic & Cloutier (2003)
P20	AX Ax2*, Ax1	F:5'-ATGACTAAGCGGTTGGTTCTT-3' R:5'-GACCTTGCTCCCCTTGTCTTT-3'	1319	Ma *et al.* (2003)
P21	AX Ax2*, Ax1	F:5'-ATGACTAAGCGGTTGGTTCTT-3' R:5'-GACCTTGCTCCCCTTGTCCTG-3'	1500	Ma *et al.* (2003)
P22	Dy10	F:5'-GACAGTCCACCGAGATGG-3' R:5'-GCAAGCTGCAGAGAGTTC-3'	1400, 2000	Mishra *et al.* (2009)
P23	Dx5	F:5'-CATGGTCCTGAACCTTCACC-3' R:5'-CAGAGAGTTCTATCACTGGC-3'	2000	Mishra *et al.* (2009)
P24	1Ax1 (Ax2)	F:5'-CCGAGATGACTAAGCGG-3' R:5'-GCTAACATGGTATGGGCT-3'	1800, 2500	Mishra *et al.* (2009)
P25	Dx5 Dx2	F:5'-CGTCCCTATAAAAGCCTAG-C-3' R:5'-AGTATGAAACCTGCTGCGGAC-3'	478	Ma *et al.* (2003)
P26	Dx5 Dx2	F:5'-CGTCCCTATAAAAGCCTAG-C-3' R:5'-AGTATGAAACCTGCTGCGGAG-3'	450	Ma *et al.* (2003)
P27	Dx5 Dx2	F:5'CGTCCCTATAAAAGCCTAGTT-3' R:5'-AGTATGAAACCTGCTGCGGAC-3'	450	Ma *et al.* (2003)
P28	Dx5 Dx2	F:5'-CGTCCCTATAAAAGCCTAGTT-3' R:5'-AGTATGAAACCTGCTGCGGAG-3'	450	Ma *et al.* (2003)
P29	1Dy10.1	F:5'-ATGGCTAAGCGGC/TTA/GGTCCTCTTTG-3' R:5'-CTATCACTGGCTG/AGCCGACAATGCG-3'	372	Jiang *et al. (*2006)
P30	degenere primer	F:ATCACCCACAACACCGAGCA-3'G R:CTATCACTGGCTA/GGCCGACAATGCG	1800, 2000	Ma *et al.* (2003)
P31	degenere primer	F:AGGGAAAGACAATGGACATG R:TAGTTG/TCCC/TAGAGGCCTCACCTTC	1800, 2000, 2100, 2500	Jiang *et al.* (2006)

Materials and Methods

Plant materials

116 bread wheat landraces of Turkey obtained from Ankara Field Crops Central Research Institute and Izmir Aegean Agricultural Research Institute were included in the study (Table 1). The specimens were so selected to provide representation of all geographical regions (Aegean, Central Anatolia, Marmara, the Mediterranean, Black Sea, Eastern Anatolia and Southeastern Anatolia) in the country. These were supplied from the "Field Crops Central Research Institute, Ankara" and "Aegean Agricultural Research Institute, İzmir. 15 standard genotypes (Chinese Spring, Drago, Lobeiro, Svevo, Lira, Durambo, Ak 702, Bezostaya 1, KateA1, Bayraktar, Mizrak, Yakar, Atay 85, Gerek 79 and Tosunbey) were used as references to define gluten alleles The wheat specimens studied with their respective distribution regions is provided in Table 1.

DNA extraction and PCR analysis

DNA was isolated from the seeds according to the method described by McCarthy *et al.* (2002) using 31 primer pairs (Table 2). PCR reaction was realized in 15 µl reaction mixture containing 50 nmol of each primer, 0.3 nmol dNTP, 1-2 unit Taq polymerase, 1.5-2.5 mM $MgCl_2$, 10 µg/µl BSA and 30 ng template DNA. Following the initial denaturation at 94°C for 5 minutes, PCR reaction was carried out in 35 cycles of 94°C for 1 minute, 54-65°C for 1-2 minutes and 72°C for 2 minutes. The last extension step was realized at 72°C for 10 minutes. PCR products were run in 1-1.5% agarose-containing ethidium bromide and imaged with a gel documentation system (BioRad MP5 gel documentation system).

Gluten extraction and SDS-PAGE analyses

Gluten proteins were extracted in accordance with the methods described by Gao *et al.* (2012) and Temizgul *et al.* (2018). The methods of Li *et al.* (2012) with slight modification were used for the electrophoresis of gluten proteins. 30-60 mA current was applied per gel during electrophoresis. Gels were stained (dissolved in 187.5 ml of methanol, 225 mg of CBB-R 250; 750 ml 10% TCA and 62.5 ml glacial acetic acid) for 12-24 hours and kept in washing solution (333 ml methanol, 100 ml 10% TCA and 567 ml distilled water) for 5 hours and imaged under the gel documentation system.

Definition of gluten alleles

Gluten alleles of the wheat landraces were defined based on individual HMW subunit distributions in SDS-PAGE in accordance with the methods specified by Payne & Lawrence (1983) and McIntosh *et al.* (1994). The verification of alleles defined by allele-specific primers was also performed.

Calculation of Glu-1 quality scores

Based on the SDS-PAGE profile, relationships between individual HMW subunits and quality was determined by using the method described by Payne (1987a) and scored by using subunit scores of Payne (1987a) and Lukow *et al.* (1989).

POPGENE version 1.31 software was used to draw dendrogram based on Nei's original measurement showing the relationships among the genotypes based on individual subunits (Nei 1972).

Statistical analysis

Data analysis was carried out based on the frequencies of HMW gluten gene alleles. Statistical analyses were performed separately in individual variety and populations for individual alleles, individual sub-units, loci (*Glu-A1*, *Glu-B1*, and *Glu-D1*) and geographical origin (7 regions). POPGENE version 1.31 software was used for statistical analysis. Polymorphism percentages were calculated using Equation 1;

Eq. 1: Polymorphism% = (number of polymorphic allele/number of total alleles) × 100.

Allele frequency of *Glu-1* loci (*Glu-A1*, *Glu-B1*, and *Glu-D1*) was calculated according to Gupta *et al.* (1991). Percentage allele frequencies were calculated by using Equation 2;

Eq. 2: Allele frequency = (number of individual alleles/number of total wheat samples) × 100.

Allelic combination frequencies were calculated using Equation 3;

Eq. 3: Allelic combination frequency = (observed total number of each allelic combinations/number of total wheat samples) × 100.

Results

In A genome of studied genotypes, the frequencies of *Glu-A1*c, *Glu-A1*b, and *Glu-A1*a were calculated to be 65.1%, 26.4%, and 8.5%, respectively (Table 3). In B genome, the frequencies of *Glu-B1*b, *Glu-B1*e, *Glu-B1*d, and *Glu-B1*c were determined to be 44.6%, 16.6%, 10.0%, and 10.7%, respectively. In D genome, the frequencies of *Glu-D1*a, *Glu-D1*d, and *Glu-D1*b were determined to be 59.7%, 27.9%, and 7.7%, respectively. Since quality scores were not determined for these subunits, the contributions of the alleles *Glu-B1*h, *Glu-B1*z, and *Glu-B1*aj to quality score could not be calculated for 7 of the 116 genotypes studied. After determination of rheological characteristics of individual subunits, the contribution of these subunits to the quality can also be determined. The average quality score in the genotypes was calculated to be 6.07. The Marmara and the Southeastern Anatolia regions had 7.18 and 4.8 quality scores as the highest and the lowest, respectively.

Subunit frequencies

Total and region-based subunit frequencies were given in Table 3. The highest frequencies were observed as 65.11%, 54.30%, and 58.30% for the subunits *Glu-A1*c, *Glu-B1*b, and *Glu-D1*a, respectively. *Glu-1* genome subunit frequencies are given in Table 3. Based on the

regions, the frequencies of *Glu-A1a* (18.2%), *Glu-A1b* (50.0%), *Glu-A1c (*80.0%), *Glu-B1b* (58.0%), *Glu-B1e* (46.0%), *Glu-D1a* (86.7%), *Glu-D1d* (50.0%), were the highest in Mediterranean, Marmara, Aegean, Black Sea, Southeastern Anatolia, Aegean and Marmara, respectively.

Table 3. Total and region-based subunit frequencies (%).

Subunits \ Regions		Marmara	Aegean	Mediterranean	Central Anatolia	Black Sea	Eastern Anatolia	South Eastern Anatolia	Total Frequency
	Glu-A1								
a	1	14.2	13.3	18.2	-	15.8	3.3	8.3	8.5
b	2*	50.0	6.7	18.2	32.1	31.6	23.3	16.7	26.4
c	Null	35.8	80.0	63.6	67.9	52.6	73.3	75.0	65.1
	Glu-B1								
a	7	7.0	5.0	-	-	-	-	-	1.7
b	7+8	27.5	56.0	17.0	52.0	58.0	56.0	46.0	44.6
c	7+9	32.0	-	8.0	14.0	9.0	3.3	8.0	10.7
d	6+8	-	24.0	8.0	31.0	4.0	3.0	-	10.0
e	20	13.0	5.0	17.0	3.0	20.0	12.3	46.0	16.6
f	13+16	-	-	-	-	-	6.0	-	0.9
h	14+15	-	-	50.0	-	-	3.3	-	7.6
I	17+18	-	5.0	-	-	-	-	-	0.7
u	7*+8	-	-	-		-	6.7	-	1.0
aj	8	7.0	5.0	-		9.0	-	-	3.0
z	7+15	13.5	-	-		-	9.4	-	3.2
	Glu-D1								
a	2+12	50.0	86.7	45.5	67.9	63.2	40.0	58.3	58.3
b	3+12	-	6.7	22.0	10.7	-	10.0	8.3	8.2
c	4+12	-	-	10.5	3.6	5.3	3.3	8.3	4.3
d	5+10	50.0	6.7	22.0	17.9	31.6	40.0	25.0	27.6
h	2+12*	-	-	-	-	-	6.7	-	1.6

Table 4. Allelic combinations and the frequencies.

Allelic combinations	Subunit combinations	Frequency (%)	Allelic combinations	Subunit combinations	Frequency (%)
*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	22.48	*Glu-A1*b/*Glu-B1*d/*Glu-D1*c	(2*, 6+8, 4+12)	0.78
*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(Null, 7+8, 2+12)	11.70	*Glu-A1*a/*Glu-B1*c/*Glu-D1*a	(1, 7+9, 2+12)	0.78
*Glu-A1*b/*Glu-B1*c/*Glu-D1*d	(2*, 7+9, 5+10)	9.35	*Glu-A1*b/*Glu-B1*c/*Glu-D1*b	(2*, 7+9, 3+12)	0.78
*Glu-B1*d/*Glu-D1*a	(6+8, 2+12)	5.45	*Glu-B1*z/*Glu-D1*d	(7+15, 5+10)	0.78
*Glu-A1*b/*Glu-B1*b/*Glu-D1*d	(2*, 7+8, 5+10)	4.70	*Glu-B1*e/*Glu-D1*d	(20, 5+10)	0.78
*Glu-B1*b/*Glu-D1*d	(7+8, 5+10)	4.70	*Glu-A1*c/*Glu-B1*b/*Glu-D1*d	(Null, 7+8, 5+10)	0.78
*Glu-A1*c/*Glu-B1*d/*Glu-D1*a	(Null, 6+8, 2+12)	3.15	*Glu-B1*e/*Glu-D1*c	(20, 4+12)	0.78
*Glu-B1*e/*Glu-D1*a	(20, 2+12)	3.15	*Glu-B1*b/*Glu-D1*c	(7+8, 4+12)	0.78
*Glu-A1*b/*Glu-B1*d/*Glu-D1*b	(2*, 6+8, 3+12)	2.35	*Glu-A1*b/*Glu-B1*e/*Glu-D1*d	(2*, 20, 5+10)	0.78
*Glu-A1*a/*Glu-B1*e/*Glu-D1*a	(1, 20, 2+12)	2.35	*Glu-A1*b/*Glu-B1*i/*Glu-D1*d	(2*, 17+18, 5+10)	0.78
*Glu-A1*b-c/*Glu-B1*b/*Glu-D1*b	(2*, 7+8, 3+12)	1.56	*Glu-A1*a/*Glu-B1*aj/*Glu-D1*a	(1, 8, 2+12)	0.78
*Glu-A1*c/*Glu-B1*z/*Glu-D1*a	(Null, 7+15, 2+12)	1,56	*Glu-A1*a/*Glu-B1*f/*Glu-D1*a	(1, 13+16, 2+12)	0.78
*Glu-B1*z/*Glu-D1*a	(7+15, 2+12)	1.56	*Glu-A1*a/*Glu-B1*aj-e/*Glu-D1*d	(1, 8, 20, 5+10)	0.78
*Glu-B1*b/*Glu-D1*b	(7+8, 3+12)	1.56	*Glu-A1*a/*Glu-B1*e/*Glu-D1*a	(1, 20, 2+12)	0.78
*Glu-B1*u/*Glu-D1*a	(7*+8, 2+12)	1.56	*Glu-A1*a/*Glu-B1*z/*Glu-D1*a	(1, 7+15, 2+12)	0.78
*Glu-A1*c/*Glu-B1*e/*Glu-D1*d	(Null, 20, 5+10)	1.56	*Glu-A1*b/*Glu-B1*d/*Glu-D1*d	(2*, 6+8, 5+10)	0.78
*Glu-A1*c/*Glu-B1*e/*Glu-D1*a	(Null, 20, 2+12)	1.56	*Glu-A1*b/*Glu-B1h*/*Glu-D1*d	(2*, 14+15, 5+10)	0.78
*Glu-A1*b/*Glu-B1*b/*Glu-D1*c	(2*, 7+8, 4+12)	1.56	*Glu-A1*b/*Glu-B1*f/*Glu-D1*d	(2*, 13+16, 5+10)	0.78
*Glu-A1*c/*Glu-B1*b/*Glu-D1*b	(Null, 7+8, 3+12)	1.56	*Glu-A1*a/*Glu-B1*f	(1, 13+16)	0.78
*Glu-A1*b/*Glu-B1*a/*Glu-D1*d	(2*, 7, 5+10)	0.78			

Table 5. Region-based allelic combinations and their frequencies.

Region	Allelic combinations	Subunit combinations	Frequency (%)	Glu-1 Score	Average Glu-1 score
Marmara	*Glu-A1*b/*Glu-B1*c/*Glu-D1*d	(2*, 7+9, 5+10)	35.71	9	7.18 ± 2.34
	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	14.29	5	
	*Glu-A1*b/*Glu-B1*a/*Glu-D1*d	(2*, 7, 5+10)	7.14	9	
	*Glu-B1*e/*Glu-D1*a	(20, 2+12)	7.14	3	
	*Glu-A1*a/*Glu-B1*e/*Glu-D1*a	(1, 20, 2+12)	7.14	6	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(null, 7+8, 2+12)	7.14	6	
	*Glu-B1*z/*Glu-D1*a	(7+15, 2+12)	7.14	?	
Aegean	*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(null, 7+8, 2+12)	26.67	6	5.10 ± 1.42
	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	20.00	5	
	*Glu-A1*c/*Glu-B1*d/*Glu-D1*a	(null, 6+8, 2+12)	13.33	3	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*b	(null, 7+8, 3+12)	6.67	6	
	*Glu-A1*a/*Glu-B1*aj/*Glu-D1*a	(1, 8, 2+12)	6.67	?	
Mediterranean	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	18.18	5	5.70 ± 2.14
	*Glu-B1*b/*Glu-D1*b	(7+8, 3+12)	18.18	5	
	*Glu-A1*b/*Glu-B1*d/*Glu-D1*d	(2*, 6+8, 5+10)	9.09	8	
	*Glu-A1*b/*Glu-B1*c/*Glu-D1*d	(2*, 7+9, 5+10)	9.09	9	
	*Glu-B1*b/*Glu-D1*c	(7+8, 4+12)	9.09	4	
	*Glu-B1*e/*Glu-D1*a	(20, 2+12)	9.09	3	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(null, 7+8, 2+12)	9.09	6	
Central Anatolia	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	39.29	5	5.63 ± 2.38
	*Glu-B1*d/*Glu-D1*a	(6+8, 2+12)	14.29	3	
	*Glu-A1*b/*Glu-B1*c/*Glu-D1*d	(2*, 7+9, 5+10)	14.29	9	
	*Glu-A1*b/*Glu-B1*b/*Glu-D1*b	(2*, 7+8, 3+12)	10.71	6	
	*Glu-A1*c/*Glu-B1*d/*Glu-D1*a	(null, 6+8, 2+12)	7.14	4	
	*Glu-A1*b/*Glu-B1*b/*Glu-D1*d	(2*, 7+8, 5+10)	3.57	10	
	*Glu-A1*b/*Glu-B1*b/*Glu-D1*c	(2*, 7+8, 4+12)	3.57	7	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(null, 7+8, 2+12)	3.57	6	
Black Sea	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	26.32	5	6.65 ± 1.73
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(null, 7+8, 2+12)	21.05	6	
	*Glu-A1*b/*Glu-B1*c/*Glu-D1*d	(2*, 7+9, 5+10)	5.26	9	
	*Glu-B1*b/*Glu-D1*d	(7+8, 5+10)	5.26	7	
	*Glu-A1*b/*Glu-B1*b/*Glu-D1*d	(2*, 7+8, 5+10)	5.26	10	
	*Glu-A1*b/*Glu-B1*d/*Glu-D1*c	(2*, 6+8, 4+12)	5.26	5	
	*Glu-A1*c/*Glu-B1*aj-e/*Glu-D1*a	(null, 8, 20, 2+12)	5.26	?	
	*Glu-A1*a/*Glu-B1*c/*Glu-D1*a	(1, 7+9, 2+12)	5.26	7	
	*Glu-A1*b/*Glu-B1*e/*Glu-D1*d	(2*, 20, 5+10)	5.26	8	
	*Glu-A1*a/*Glu-B1*e/*Glu-D1*a	(1, 20, 2+12)	5.26	6	
Easter Anatolia	*Glu-B1*b/*Glu-D1*d	(7+8, 5+10)	16.67	7	6.57 ± 2.10
	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	10.00	5	
	*Glu-B1*u/*Glu-D1*a	(7*+8, 2+12)	6.67	5	
	*Glu-A1*c/*Glu-B1*e/*Glu-D1*a	(null, 20, 2+12)	6.67	4	
	*Glu-A1*c/*Glu-B1*h/*Glu-D1*a	(null, 14+15, 2+12)	3.33	?	
	*Glu-A1*b/*Glu-B1*b/*Glu-D1*d	(2*, 7+8, 5+10)	3.33	10	
	*Glu-A1*b/*Glu-B1*b/*Glu-D1*b	(2*, 7+8, 3+12)	3.33	7	
	*Glu-A1*c/*Glu-B1*d/*Glu-D1*a	(null, 6+8, 2+12)	3.33	4	
	*Glu-A1*c/*Glu-B1*z/*Glu-D1*a	(null, 7+15, 2+12)	3.33	?	
	*Glu-B1*z/*Glu-D1*h	(7+15, 2+12*)	3.33	?	
	*Glu-B1*z/*Glu-D1*d	(null, 7+15, 5+10)	3.33	?	
	*Glu-B1*b/*Glu-D1*h	(7+8, 2+12*)	3.33	5	
	*Glu-A1*b/*Glu-B1*c/*Glu-D1*d	(2*, 7+9, 5+10)	3.33	9	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*d	(null, 7+8, 5+10)	3.33	8	
	*Glu-A1*b-c/*Glu-B1*f/*Glu-D1*d	(2*, 13+16, 5+10)	3.33	10	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*b	(null, 7+8, 3+12)	3.33	6	
	*Glu-A1*c/*Glu-B1*e/*Glu-D1*d	(null, 20, 5+10)	3.33	6	
South Eastern Anatolia	*Glu-B1*b/*Glu-D1*a	(7+8, 2+12)	16.67	5	4.80 ± 1.77
	*Glu-B1*e/*Glu-D1*a	(20, 2+12)	16.67	3	
	*Glu-A1*c/*Glu-B1*b/*Glu-D1*a	(null, 7+8, 2+12)	16.67	6	
	*Glu-B1*e/*Glu-D1*c	(20, 4+12)	8.33	2	
	*Glu-A1*b/*Glu-B1*c/*Glu-D1*b	(2*, 7+9, 3+12)	8.33	7	
	*Glu-A1*c/*Glu-B1*e/*Glu-D1*d	(null, 20, 5+10)	8.33	6	
	*Glu-B1*e/*Glu-D1*d	(20, 5+10)	8.33	5	

Allelic combinations and frequencies

Allelic combinations and their frequencies are given in Table 4. The results indicated the presence of 39 different allelic combinations. Among these combinations, *Glu-B1*b/*Glu-D1*a was the most frequent combination with a 22.48% frequency value, followed by *Glu-A1*c/*Glu-B1*b/*Glu-D1*a, *Glu-A1*b/*Glu-B1*c/*Glu-D1*d, and *Glu-B1*d/*Glu-D1*a allelic combinations with 11.7%, 9.35%, and 5.45% frequencies, respectively. The rest of the alleles were observed with a frequency less than 5%.

Region-based allelic combinations and Glu-1 scores

The frequencies of HMW gluten subunits and their *Glu-1* scores are given in Table 5. In some of the genotypes, the effect of some subunits on quality score could not be calculated since the effect of these subunits on quality score was not determined. The quality score was 10 in four accessions (TR32846-6, TR36948-1, TR45105 and TR63536), and 9 in 6 accessions (TR45398-4, TR48025-3, TR33264-6, 393-5, TR52021-3, TR45094). The highest (17) and the lowest (5) allelic combinations were observed in Eastern Anatolia and Aegean regions, respectively (Table 5).

Development of dendrogram based on subunits of Glu-1 loci

When the dendrograms (Nei 1972) drawn based on individual subunits were investigated, the Mediterranean region clustered with Black sea (genetic distance 0.32) and Aegean region clustered with Central Anatolia (genetic distance 0.52) (Fig. 1). Marmara (genetic distance 1.35), Eastern Anatolia (genetic distance 0.37), and Southeastern Anatolia (genetic distance 0.52) regions were separately clustered.

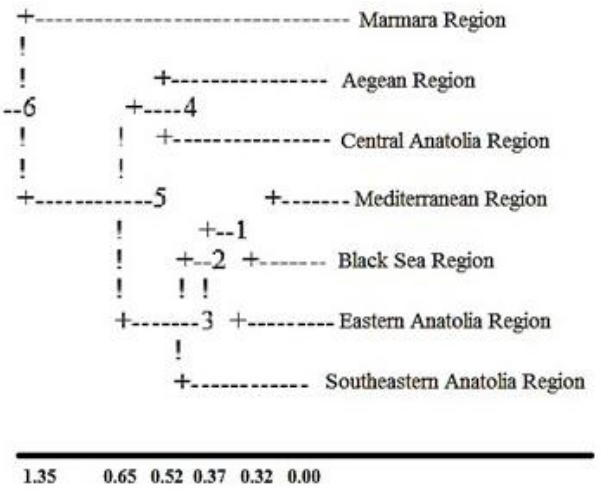

Fig. 1. Dendrogram showing the relationships of the genotypes based on individual subunits of *Glu-1* loci.

Discussion

The determination of HMW-GS composition in wheat cultivar collections from different countries has been studied (Nucia *et al.* 2019). The contribution of individual subunits on dough quality is quite different (Payne *et al.* 1987, Lukow *et al.* 1989). While quality score of *Glu-D1*d is the highest (quality score 4), the contribution of *Glu-A1*c, *Glu-B1*a, *Glu-B1*d, *Glu-B1*e, and *Glu-D1*c is considered to be the lowest (quality score 1). In the present study, the highest *Glu-D1*d frequency (50%) was observed in Marmara and the lowest was observed in Aegean regions (6.67%). The frequency of this subunit was 27.6% throughout Turkey (Table 2). The highest *Glu-1* score with 7.18 was observed in Marmara followed by Black Sea with 6.65, Eastern Anatolia with 6.57, Mediterranean with 5.70, Central Anatolia with 5.63, Aegean with 5.10, and Southeastern Anatolia regions with 4.8 (Table 5).

Observing the highest quality score in Marmara and the lowest in the Southeastern region is an expected outcome. Southeastern Turkey is on the interception of gene centers for wheat and its wheat is intensively studied and high-quality score wheat genotypes are already selected for breeding. Marmara region is the most industrialized region and agricultural activity is quite poor in this region, therefore, the landraces might not be used to select for high-quality score cultivar breeding. Throughout Turkey, 19 different subunits (3 *Glu-A1*, 11 *Glu-B1* and 5 *Glu-D1*) were observed in 39 different combinations (Tables 3 and 4). Among the allelic combinations *Glu-B1*b/*Glu-D1*a was the highest with 22.48% frequency, followed by *Glu-A1*c/*Glu-B1*b/*Glu-D1*a with 11.70%, *Glu-A1*b/*Glu-B1*c/*Glu-D1*d with 9.35% and *Glu-B1*d/*Glu-D1*a with 5.45%. Other alleles were observed with less than 5% frequency. The highest allelic variation with 16 subunits and 17 different combinations was observed in Southeastern Anatolia. *Glu-B1*b/*Glu-D1*d allelic combinations with 16.67% frequency were the most frequent allelic combinations in this region (Table 5). Based on Nei's original measurement (Nei 1972), while the highest similarity among regions for individual subunits of *Glu-1* loci was observed between Black Sea and the Mediterranean regions (0.9935), the lowest similarity was observed in Marmara and the Aegean regions (0.953). When the dendrogram was drawn based on Nei's (1972) original measurements, the Mediterranean clustered with the Black Sea and the Aegean with the Central Anatolia accessions. Other regions were separately clustered (Fig. 1).

Nakamura *et al.* (1999) studied variations in the HMW subunits of *Glu-1* loci of Kapon wheats and identified 14 different alleles, 3 of which were on *Glu-A1*, 6 on *Glu-B1* and 5 on *Glu-D1* loci. In the present study, 19 different subunits were identified and 3 of them were on *Glu-A1*, 11 of them on *Glu-B1* and 5 of them on *Glu-D1* loci. The frequency of null alleles located on chromosome 1A was reported to be high (74%) on Japanese wheat especially on Norin variety. The frequency of this allele was also found to be high in landraces of Turkish wheat (65.11 %). While *Glu-B1*a, *Glu-B1*f, *Glu-B1*h, *Glu-B1*j, and *Glu-B1*k subunits were not observed in Japanese bread wheat (Nakamura *et al.* 1999), only *Glu-B1*j and *Glu-B1*k

subunits were not observed in Turkish wheat genotypes used in this study. While the frequency of 2+12 subunit coded by *Glu-D1*a allele was 55% in Japanese wheat, it was only 1.5% in 5+10 subunit coded by *Glu-D1*d. The average frequencies of these alleles were found to be 58.3% and 27.6% in Turkish genotypes, respectively. The frequencies of *Glu-D1*a and *Glu-D1*d subunits were 86.7% and 50% in Aegean and Marmara regions, respectively. The frequency of *Glu-D1d* subunit was found to be high in European wheat varieties (Payne *et al.* 1984). The frequency of this subunit was also found to be high in Turkish wheat landraces, especially in Marmara region (average 27.6%, Marmara region 50.0%). Cabellero *et al.* (2009) investigated the seed storage protein diversity of the wild diploid wheat genotypes obtained from Lebanon and Turkey. They determined 10 alleles at *Glu-A1*, 16 alleles at *Glu-A3*, 15 alleles at *Gli-A1*, 18 alleles at *Gli-A2* and detected 4 frequent, 2 infrequent, and 3 rare alleles. In the present study, 11 frequent, 7 infrequent and 4 rare alleles were determined.

Van Hintum & Elings (1991) evaluated the Syrian durum wheat genotypes based on phenotype and gluten content. They observed 19 HMW subunits in 48 different combinations. In the present study, 22 individual alleles and 19 HMW subunits were detected in 39 different combinations. Gianibelli *et al.* (2002) studied molecular and biochemical characterizations of Argentinian wheat cultivars, identified the allelic variations, and calculated the allele frequencies. Of the 11 alleles, 3 were coded by *Glu-A1*, 6 were coded by *Glu-B1* and 2 were coded by *Glu-D1* loci. Null allele frequency was found to be quite low (1.1%) and *Glu-D1*d was the highest in frequency. However, *Glu-A1*c subunit was the highest with the frequency of 65.1% among the alleles in Turkish landraces. Gianibelli *et al.* (2002) also calculated the quality score taking the *Glu-A1* into account and separated the Argentinian wheat into 18 groups. *Glu-A1*a/*Glu-B1*bq/*Glu-D1*d was observed in highest frequency (22%). In Turkish landraces, *Glu-B1*b/*Glu-D1*a was observed in highest frequency.

Payne (1987b) found the quality score of world wheat collection as 9.5. The quality score of Turkish wheat landraces was found to be significantly lower (6.07). Notwithstanding, quality score is not determined by only HMW-GS, the contribution of LMW-GS and Gliadins should also be taken into account (MacRitchie *et al.* 1990).

Payne & Lawrence (1983) published the catalogue of *Glu-1* alleles. They determined 3, 11, and 7 alleles in *Glu-A1, Glu-B1*, and *Glu-D1,* respectively. Additional alleles were also determined but most of those alleles were found to be in *Glu-B1* loci (Pogna *et al.* 1990). In the present study, a possible new allele (Dy12*) was determined in *Glu-D1* locus. This new subunit was differentiated considering its faster movement in SDS-PAGE.

Branlard *et al.* (1989) observed 3 allelic variations in *Glu-A1* of 165 Turkish durum wheat cultivars. *Glu-A1*-1 allele was coding an x subunit that had bigger electrophoretic mobility than 2*. The researchers suggested that this allele was similar to previously discovered two alleles (*Glu-A1*V, *Glu-A1*VI). The null allele with 68.9% frequency was the most frequent allele followed by *Glu-A1*b (28.3%) and *Glu-A1*-1 (3.8%). Seven different *Glu-B1* allele variants were identified in previous studies subjecting the Turkish wheats (Branlard et al. 1989) . These consisted of 5 different x and y type subunit combinations. Of the 7 *Glu-B1*, 5 (*Glu-B1*b, *Glu-B1*d, *Glu-B1*e, *Glu-B1*h, and *Glu-B1*z) were observed among Turkish wheat samples (Branlard *et al.* 1989, Payne *et al.* 1981). In the present study, 11 subunits were determined in *Glu-B1* loci (Table 3).

Primitive cultivars and locally grown landraces were considered to be the sources of variation for grain protein quality, disease resistance, and resistance to abiotic stress conditions (Porceddu *et al.* 1988, Kaplan *et al.* 2014). HMW subunit variations in Turkish bread wheat landraces were found to be higher compared to Australian, Italian, American, Canadian, French and Spanish wheat samples (Autran & Feillet 1985, Margiotta *et al.* 1987, Carrillo *et al.* 1990). This outcome is somewhat expected since intensive breeding works have decreased the variation in western wheat genotypes (Porceddu *et al.* 1988).

Morgunov *et al.* (1993) and Sultana *et al.* (2007) stated that the increase in HMW score was related to a decrease in diversity in gluten alleles. A similar phenomenon was also observed in wheats grown in Dobrudzha Agricultural Institute (Atanasova *et al.* 2009). That is why it is crucial to use *Glu-B1*f, *Glu-B1*h and *Glu-B1*i subunits to increase quality score. This situation may decrease genetic diversity and increase end-product quality (Liu *et al.* 2007). In the present study, the observation of these alleles was also quite low in 116 Turkish wheat landraces (approximately 7%). The frequency of *Glu-B1*h (14+15) was observed to be 55% in the Mediterranean region. Maintenance of *Glu-A1*b (2*) and *Glu-D1*d (5+10) alleles is important. These alleles contribute to quality supported with *Glu-B1*i (17+18), *Glu-B1*f (13+16) and *Glu-B1*h (14+15) (Tsenov *et al.* 2009).

Terasawa *et al.* (2010) studied the genetic variation of high molecular weight gluten subunits and identified 3, 9 and 15 alleles in *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively. *Glu-A1*c (74.4%), *Glu-B1*b (76.5%) and *Glu-D1*a (81.5%) were the most frequently observed alleles. Although *Glu-D1*a (46.9%) was the most frequently observed allele in Central Asia, it was lower in all the other regions except Caucasian region. A total of 83 allelic combinations were determined on *Glu-1* loci in their studies. Among the allelic combinations, *Glu-A1*c/*Glu-B1*b/*Glu-D1*a was the most frequently observed genotype. The frequency of this allelic combination was found to be 11.70% for Turkish cultivars. The most frequently observed allelic combination was *Glu-B1*b/*Glu-D1*a (22.48%). Although western Asian, Afghanistan, and Eastern Asian wheats were exhibiting

similar characteristics, Caucasian and Central Asian wheats differed from these three regions. As it is reported by Terasawa *et al.* (2009) and Lagudah *et al.* (1987), the most common genotypes were determined to be *Glu-A1*c (null), *Glu-B1*b (7+8), and *Glu-D1*a (2+12) among Western and Eastern Asian genotypes. These alleles were also found to be the highest in frequency in the present study. Those results, the results of Terasawa *et al*. (2009) and Lagudah *et al.* (1987) indicated that *Glu-A1*c, *Glu-B1*b, and *Glu-D1*a genotypes were dominant in regions extending from Mesopotamia, Afghanistan, and Far East to Central Asia. In Southern Asia, *Glu-A1*c, *Glu-B1*i, and *Glu-D1*a were the most frequently observed genotypes in a study reported by Terasawa *et al.* (2010). This genotype was considered to be a modified version of the typical Asian genotype in the sense that only *Glu-B1*i allele was replacing *Glu-B1*b allele. *Glu-B1*i allele was observed rarely in other regions of Southern Asia. This allele was also found to be very rare in the present study (0.78%). Similarly, this allele was also rare among European endemic wheat genotypes (Gregova *et al.* 1999, 2006, Juha'sz *et al.* 2003). This is why researchers considered that *Glu-B1*i had appeared in Southern Asia (Terasawa *et al.* 2010). *Glu-B1*i allele was providing more firmness to dough compared to *Glu-B1*b allele (Payne & Lawrence 1983, Mondal *et al.* 2008). *Glu-D1*d allele is common in Caucasian and Central Asian accessions. The high frequency of this allele in Caucasian and Central Asia is remarkable. In the same region *Glu-A1*b and *Glu-B1*a alleles have also been observed in high frequency (Terasawa *et al.* 2010). *Glu-D1*d allele is known to contribute to bread-making quality. This allele is introduced to modern wheat genotypes to increase bread-making quality (Wrigley *et al.* 2015, online). The frequency of this allele was calculated to be 27.91% in the present study and the allele was observed in 50% of the wheat genotypes. This allele was also in high frequency in European wheat (Gregova *et al.* 1999, 2006, Juha'sz *et al.* 2003). However, researchers suggested the Caucasian region as the center of origin for this allele and its dispersion to other regions (Dvorak *et al.* 1998).

In conclusion, the quality score was found to be low in the studied genotypes (6.07). High quality score genotypes might have already been selected for breeding purposes by agricultural institutes. Relatively high quality scores in Black Sea region, where wheat breeding studies is relatively low, and in highly industrialized Marmara region support that claims. Considering the individual alleles in *Glu-1* loci, the highest similarity was observed between Black Sea and the Mediterranean regions (0.9953) and the lowest similarity was between Marmara and Aegean regions (0.9472). When individual alleles and subunits are used for cluster analysis in *Glu-1* loci, the Mediterranean region clustered with Black Sea and Aegean region clustered with Central Anatolia. Other regions are individually clustered and separated from these regions.

Among the studied genotypes, 4 accessions (TR32846-6, TR36948-1, TR45105 and TR63536) were determined to be reaching to the highest score (quality score 10). Of the 116 studied accessions, 6 genotypes (TR45398-4, TR48025-3, TR33264-6, TR393-5, TR52021-3 and TR45094) had the quality score of 9. To investigate new *Glu-1* alleles, more landraces need to be studied. To verify new putative alleles, 2D gel electrophoresis and peptide sequencing could also be applied in addition to PCR and SDS-PAGE.

Although we detected 50 different allelic combinations among the studied accessions, we were able to calculate the quality score of 39 accessions. Glu-B1b/Glu-D1a with 22.48% frequency was the most frequent combination. This was followed by Glu-A1c/Glu-B1b/Glu-D1a, Glu-A1b/Glu-B1c/Glu-D1d and Glu-B1d/Glu-D1a allelic combinations with 11.7%, 9.35% and 5.45% frequencies, respectively. The highest quality scores were observed in Glu-A1b/Glu-B1c/Glu-D1d (quality score 10 with 4.70% frequency) and in Glu-A1b/Glu-B1b/Glu-D1d (quality score 9 with 9.35% frequency) allelic combinations, respectively.

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Research Article

EVALUATION OF ANTICANDIDAL EFFECTS OF ESSENTIAL OILS OF COMMERCIAL LAVENDER (*Lavandula angustifolia* Miller) IN COMBINATION WITH KETOCONAZOLE AGAINTS SOME *Candida* Berkhout STRAINS

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Abstract: Application of combination studies with essential oils and standard antifungal drugs may reduce adverse effects of synthetic drugs and serve as alternative approach against various pathologies including candidiasis. The aim of the present study was to determine the interaction of two commercial lavender (*Lavandula angustifolia* Miller) essential oils in combination with ketoconazole against clinical and standard strains of the human pathogens *Candida albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032. The chemical compositions of the investigated essential oils were confirmed both by gas chromatography/flame ionization detector (GC/FID) and gas chromatography/mass spectrometry (GC/MS) methods. Anticandidal activities of the essential oils were screened *in vitro* by the microdilution method. The resulting interaction of combining lavender essential oils and ketoconazole was tested using the checkerboard method. The results showed that the interaction between essential oils and ketoconazole revealed additive and indifferent effects against the tested strains. In conclusion, the effects observed by the combination of lavender essential oils and ketoconazole may be encouraging to be used against pathogenic *Candida* strains.

Özet: Uçucu yağların standart antifungal maddeler ile kombinasyonları, sentetik ilaçların olumsuz etkilerini azaltabilir ve kandidiyaz dahil olmak üzere çeşitli patolojilere karşı alternatif bir yaklaşım olarak kullanılabilir. Bu çalışmanın amacı, Farmakope kalitesindeki Lavanta (*Lavandula angustifolia* Miller) uçucu yağlarının ketokonazol ile kombinasyonlarının patojen *Candida albicans* ATCC 10231, *C. krusei* NRRL Y-7179 ve *C. glabrata* ATCC 66032 suşlarına karşı antikandidal etkinliğini belirlemektir. İncelenen uçucu yağların kimyasal içerikleri hem gaz kromatografisi/alev iyonlaşma dedektörü (GK / AİD) hem de gaz kromatografisi/kütle spektrometresi (GK/KS) yöntemleriyle belirlenmiştir. Esansiyel yağların antikandidal aktiviteleri, mikrodilüsyon metodu ile *in vitro* olarak incelenmiştir. Lavanta uçucu yağları ve ketokonazol kombinasyon çalışmaları dama tahtası yöntemi kullanılarak test edilmiştir. Lavanta uçucu yağları ile ketokonazol kombinasyon çalışması sonuçları, test edilen tüm *Candida* suşlarına karşı "aditif" ve "indiferent" etkili olduğu görülmüştür. Sonuç olarak, lavanta uçucu yağlarının ketokonazol kombinasyonu ile gözlemlenen etkiler, patojenik *Candida* suşlarına karşı kullanımda etkili olabilir.

Introduction

Infectious diseases have recently increased the prolonged use of various antifungal drugs, contributing to the development of drug resistance against some species of the fungal genus *Candida* Berkhout, such as the azole-resistant *Candida albicans* (Robin) Berkhout strains along with *C. krusei*. In addition, many cases have been attributed to *Candida* infections particularly in persons with hematologic malignencies and transplant recipients patients (Pfaller *et al*. 2008).

The antifungal effect of azoles is due to their actions in inhibition of ergosterol biosynthesis on fungal cell membranes (Casalinuovo *et al*. 2004, Sardi *et al.* 2013). Currently used antifungal drugs may have the risk of toxicity, may result drug–drug interactions and lack of fungicidal efficacy. Furthermore, resistance to these drugs often develops rapidly (Metzger & Hofmann, 1997, Pinto *et al.* 2009).

Plants are known to produce a great variety of compounds. Particularly, aromatic plants have been used in folk medicine as antimicrobial agents since ancient times. (Hosseinzadeh *et al.* 2015). Essential oils (EOs) of many plants are known to possess a large number of pharmacologically active compounds and have antimicrobial activity. EOs show a wide-spectrum antimicrobial activity, including disruption of cell-membrane structures, leading to an alteration in membrane permeability and a consequent leakage of cell contents (Cox *et al.* 2000, Chen *et al.* 2013). For instance, recent studies on antifungal activities of some EOs have shown disruption of the fungal membrane (Pinto *et al.* 2006, Khan *et al.* 2010, Samber *et al.* 2015).

With this in mind, combination of antifungal therapies with natural sources may be achieved to overcome the resistance against *Candida* species. Specifically, EOs are known to be used in therapeutic preparations for centuries (Hosseinzadeh *et al.* 2015). The EOs of various *Lavandula* species from Lamiaceae are well known to have antimicrobial activities along with other effects they posses (Cavanagh & Wilkinson 2002, Fismer & Pilkington 2012). Thus, using the combinations of EOs with standard antifungal drugs employ as an alternative approach against various pathologies including candidiasis and may reduce adverse side effects, lower the dose and decrease toxicity (Cottarel & Wierzbowski 2007). EOs of various plants have been previously investigated for their antifungal efficacy in combination with some antifungal drugs and proved to be successful (Shin & Kang 2003, Shin 2003, Amber *et al.* 2010, Göger *et al*. 2018). Shin & Kang (2003) reported that EO fraction of *Agastache rugosa* combined with ketoconazole was found to be synergistic against the opportunistic fungus *Blastoschizomyces capitatus*. *Pelargonium graveolens* EO fractions were demonstrated to have additive effects with amphotericin B and ketoconazole against *Aspergillus* species (Shin 2003). In more recent studies, *Ocimum sanctum* EO and its combination with two azoles, fluconazole and ketoconazole, were studied against resistant isolates (Amber *et al*. 2010). *Matricaria recutita* EO was combined with fluconazole against *C. albicans* (Göger *et al*. 2018). It thus appears that, although proved to have antimicrobial activities when tested alone, studies on combination of lavender EOs with antifungal drug are rather limited. In one recent study, nystatin was tested in combination with *Lavandula angustifolia* EO against *C. albicans* ATCC 10231 and an indifference effect was observed for the combination (De Rapper *et al.* 2016)

This study was performed with the aim of identification of chemical compositions of two EOs of lavender (*L. angustifolia* Miller) by GC/FID and GC/MS analytical methods and evaluate their anticandidal activities with ketoconazole by microdilution and checkerboard methods against *C. albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032 strains.

Materials and Methods

Materials

Two commercial Lavender EOs were purchased from Sevil (Turkey) and Caesar & Lorethz GmbH (Hilden, Germany) companies and were labeled as L1 and L2, respectively. The standard powders of ketoconazole and resazurin were obtained from Sigma-Aldrich.

Anticandidal activities of the EOs of L1 and L2 were tested against standard (*C. albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032) and clinical (*C. albicans*, *C. krusei* and *C. glabrata*) isolates provided from Akdeniz University, Faculty of Medicine, Antalya.

GC/FID and GC/MS Analyses

GC /FID analysis

The EOs were analyzed by capillary GC using the Shimadzu GC 2010 system. The column and analysis conditions used for GC/MS were also applied for GC. FID detector temperature was 250°C. In order to get the same elution order with GC/MS, injections were done by using the same column and operational parameters.

GC/MS analysis

The GC/MS analysis was realized with the Shimadzu GC 2010 system. CP-Sil 5 CB column (25 m x 0.25 mm Ø, 0.25 µm film thickness) was used with helium as the carrier gas (1 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 260°C at a rate of 4°C/min, and kept constant at 260°C for 5 min. Split ratio was adjusted to 50:1. The injector temperature was at 250°C. MS were recorded at 70 eV, where the mass range was from *m/z* 35 to 450.

Identification of the volatile components

Mass spectrometry (MS) was used to characterize the volatiles by comparing their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (McLafferty & Stauffer 1989, Koenig *et al.* 2004) and *in-house* "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (Joulain & Koenig 1998, ESO (1998)) were also used for the identification.

Determination of minimum inhibitory concentration (MIC)

Anticandidal activities were performed according to the adapted guidelines of Clinical and Laboratory Standards Institute (CLSI) M27-A2 standard method. Minimum inhibitory concentrations (MIC) of EOs of L1 and L2 and ketoconazole were determined using the standard and clinically isolated *Candida* isolates (see above).

Stock solutions of EOs and ketoconazole were dissolved in DMSO. EOs were diluted two-fold initially, with a final concentration range 2560 - 5 µg/mL, whereas the ketoconazole was diluted from 64 to 0.125 µg/mL.

Candida cultures with a final inoculum size of $1x10^3$ colonies forming units (CFU/mL) were prepared. A 10 µL of the suspensions at 10^3 CFU/mL were inoculated into a two-fold diluted solutions of the tested EOs and ketoconazole in 96 well microplates. The microplates were incubated at 35°C for 48 h. Microbial growth was visualized by adding 20 µL resazurin (0.01%). MIC was defined as the lowest concentration, which did not result in any visible growth compared with the control plates. Microplates free of antifungal standards were used as the growth controls. Negative controls were used to confirm sterility and the effects of the solvents. Assays were undertaken in triplicate.

Combination of Lavender EOs and ketoconazole by the checkerboard method

The antifungal interaction between ketoconazole and EOs of L1 and L2 were studied by the checkerboard method using a 96-well plate using an 8-by-8 well configuration (Van Vuuren *et al.* 2009). EOs of L1 and L2 and ketoconazole were prepared as serial dilutions using the same solvents (medium) in the MIC test.

50 µL aliquots of each EOs were added to the wells of a 96-well plate in a vertical orientation and 50 µL aliquots of each ketoconazole dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the EOs and ketoconazole. Growth controls were applied in wells free of antifungal standards. Negative control was also applied to confirm sterility. Each well was inoculated with 100 µL (5×10^3 CFU per well) of one of the six *Candida* suspensions and incubated at 35°C for 48 hours. Following the incubation, 20 µL resazurin was added to the wells and the wells were left at 35°C for 2 h. Growth in wells was indicated by the color change from blue to pink.

In vitro interaction between Lavender EOs and ketoconazole were determined by using the fractional inhibitory concentrations (FIC). The FIC is expressed as the interaction of two compounds, where the concentration of each test compound in combination is expressed as a fraction of the concentration that would produce the same effect when used singularly (Vuuren *et al.* 2007, Stanojevic *et al.* 2010) and may be classified as follows:

$$\text{FIC of EO} = \frac{\text{MIC of EO in combination with antifungal drug}}{\text{MIC of EO alone}}$$

$$\text{FIC of ketoconazole} = \frac{\text{MIC of antifungal drug in combination with EO}}{\text{MIC of ketoconazole}}$$

The FIC index (FICI) was calculated by adding both FICs:

FICI = FIC of essential oil + FIC of ketoconazole and the FICI was interpreted as representing as;

FICI ≤ 0.5= synergism effect

FICI 0.5 ≤ 1= additive effect

FICI 1 to 4 = indifferent effect

FICI ≥ 4 = antagonism effect

Results

In our present study, lavender EOs were analyzed by GC/FID and GC/MS methods and confirmed at the quality of Pharmacopoeia grade. The EOs are characterized by high amounts of linalyl acetate (30.5-42%) and linalool (35.9- 46%) (Figures 1 and 2). The major constituents of EOs and their relative percentages are listed in Table 1. The chemical composition of lavender EOs have previously been revealed by different studies with which the EOs were reported to be generally characterized by high levels of linalool, linalyl acetate, lavandulyl acetate, terpinen-4-ol and lavandulol (Cassella *et al.* 2002, Cavanagh & Wilkinson 2002, Shellie *et al.* 2002, Dauria *et al.* 2005, Lodhia *et al.* 2009, Sokovic *et al.* 2010, Danh *et al.* 2013, Jianu *et al.* 2013, De Rapper *et al.* 2013, Yap *et al.* 2014, De Rapper *et al.* 2016, Kırımer *et al.* 2017). The amount of 1,8-cineole and camphor, on the other hand, often varies between very low to moderate (Cavanagh & Wilkinson 2002, Shellie *et al.* 2002).

Table 1. Major constituents of lavender EOs.

*KI	Compounds	L1 (%)	L2 (%)
0939	α-Pinene	-	3.2
1029	Limonene	-	trace
1031	1,8-cineole	4.8	0.7
1097	**Linalool**	**44.0**	**35.8**
1146	Camphor	4.8	0.5
1169	Borneol	1.6	2.9
1177	Terpinen-4-ol	-	1.7
1189	α-Terpineol	1.4	1.4
1257	**Linalyl acetate**	**30.5**	**36.0**
1381	Lavandulyl acetate	1.04	1.0
TOTAL	**%**	88.14	83.2

*KI: Kovats indices calculated according Adams (2001)
L1 and L2 correspond to two purchased lavender Eos
(-) Not detected

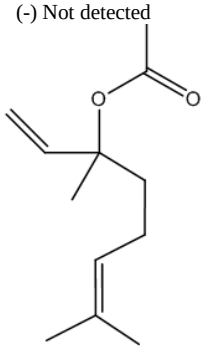

Fig. 1. Linalyl acetate

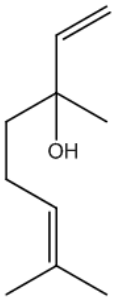

Fig. 2. Linalool

Resistance of yeast isolates to antifungal drugs, such as fluconazole and ketonazole were reported to increase in serious fungal infections making patient management a complicate case (Sanglard & Odds 2002, Jamil *et al.* 2017). *Candida species* are considered as one of the most important causes of human infections. They may become pathogenic and cause candidiasis under some conditions, such as prolonged antifungal therapy and reduced immunity. Candidiasis range from mild infection such as

onychomycosis or perlish to potentially fatal systemic candidiasis (Akortha *et al*. 2009).

Azoles are by far the most commonly used antifungals in clinical treatment with lower cytotoxicity and perfect efficacies. However, their prolonged use has led to the development of drug resistance in *Candida* species (Hasanbeigi *et al.* 2005, Magill *et al.* 2006, Zomorodian *et al.* 2011).

Many essential oils have been proven to possess antifungal properties and thus they can be potentially used as antifungal agents. As proven *in vitro*, EOs show effective antifungal activities even against resistant clinical isolates of *Candida* species. The use of combinations of EOs and their isolated components are thus new approaches to increase their efficacy in fungal treatment, for taking advantage of their synergistic and additive effects (Cleff *et al*. 2010, Pozzatti *et al.* 2010, Stringaro *et al.* 2014).

From this point of view, EOs of lavender tested for their anticandidal activities against *Candida* strains (Table 2) were found to have a MIC range of 160-640 µg/mL compared to ketoconazole MIC range of 1-4 µg/mL. In particular, the inhibitory effect of L2 EO represented with a MIC value of 160 µg/mL was more pronounced than that of L1 EO when tested with standard and clinical *C. krusei* strains (Table2).

Table 2. MIC values (µg /mL) of EOs and ketoconazole against *Candida* isolates.

Tested *Candida* isolates	MIC values of L1 EOs	MIC values of L2 EOs	MIC values of Ketoconazole
***C. albicans* ATCC 10231**	320	320	2.0
***C. krusei* NRRLY-7179**	320	160	2.0
***C. glabrata* ATCC 66032**	320	640	2.0
***C. albicans* clinical isolate**	320	320	2.0
***C. krusei* clinical isolate**	320	160	2.0
***C. glabrata* clinical isolate**	320	320	2.0

The checkerboard results of EOs and ketoconazole combinations were listed in Tables 3 and 4. The L1 + ketoconazole combination against both clinical and standard *C. albicans* ATCC 10231, *C. krusei* NRRL Y-7179 and *C. glabrata* ATCC 66032 showed additive effects (FICI= 0.53 to 056) (Table 3). The L2 + ketoconazole combination against *C. albicans* (FICI= 1.0) and *C. glabrata* (FICI= 0.56) clinical isolates also resulted in an additive activity pattern (Table 4).

In combination therapy, interactions between EOs and antifungals can display four possible types of effects as synergistic, additive, indifferent and antagonistic. Synergism is observed when the effect of the combined substances is greater than the sum of their individual effects. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Indifference is observed when there is no interaction. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when applied individually (Bassole *et al.* 2012, Yap *et al*. 2014).

The different interactions observed in antifungal activity between EOs of L1 and L2 can be explained by the chemical compositions EOS and the ratio of their components. Since EOs contain many chemical mixtures, differences can be observed in their antimicrobial effects and the type of effect mechanisms. Most of the antimicrobial activity effect for EOs was attributed to their chemical compositions derived from hydrocarbons, oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols (Burt, 2004).

Lavender EOs have been previously demonstrated to be active against a broad spectrum ranging from Gram positive and Gram negative bacteria to *Candida* strains (Dauria *et al.* 2005, Lodhia *et al* 2009, Sokovic *et al.* 2010, Danh *et al.* 2013, Jianu *et al.* 2013), and thus they were suggested to be useful in treating bacterial and fungal infections including those related with drug resistant strains. Along with their antimicrobial activity, lavender EOs were used in the treatment and relieve of symptoms of skin conditions such as psoriasis, dermatitis and eczema. It has also been suggested that topical application of lavender EOs may inhibit parts of the allergic pathway (Cavanagh & Wilkinson 2002).

Synergistic antifungal activity of combination of lavender EOs and tea tree oil (*Melaleuca alternifolia)* against dermatophyte infections were reported (Cassella *et al.* 2002). De Rapper *et al.* (2013) investigated the combination of *L. angustifolia* EO with fourty five different aroma therapeutic essential oils against *C. albicans* ATCC 10231, *P. aeruginosa* ATCC 27858 and *S. aureus* ATCC 6538. The most favourable interactions were displayed with *L. angustifolia* and *Cinnamomum zeylanicum* or with *Citrus sinensis* EOs against *C. albicans* and *S. aureus.*

L. angustifolia EO was also combined with the commercial antimicrobial drugs nystatin, chloramphenicol, ciprofloxacin and fusidic acid against *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27858 and *C. albicans* ATCC 10231. The best interaction was shown between *L. angustifolia* EO with chloramphenicol and tested against the pathogen *P. aeruginosa* (FICI= 0.29).

The mode of action of lavender EO on antimicrobial activity against multi-drug resistant *Escherichia coli* J53 R1 when used singly and in combination with piperacillin was demonstrated (Yap *et al.* 2014). The time-kill analysis showed synergistic interaction between lavender EO and piperacillin at 4 h. The results of this study indicate that lavender EO is able to disrupt membrane structures of *E. coli* and has the potential to inhibit bacterial quorum sensing.

In conclusion, we reported here for the first time the anticandidal effects of *L. angustifolia* EOs with ketoconazole combination against the standard and clinical strains of the human pathogens *C. albicans*, *C. glabrata* and *C. krusei* in a comparative manner. As revealed by the results, the absence of antagonism may be promising for combination of lavender EOs and ketoconazole in anticandidal use. The effective dose of ketoconazole use in combination may be reduced, rapid drug resistance may be prevented or combination use may provide a solution to drug-drug resistance problems and can be considered a promising alternative pharmaceutical agent for topical application in surface infections. Therefore, lavender EOs should be combined with different antibiotic/antifungal drugs and tested against different pathogens.

Table 3. Fractional inhibitory concentration index (FICI) for L1 EO.

Candida isolates	L1 MIC (alone) (µg/ml)	Ketoconazole MIC (alone) (µg/ml)	L1 MIC (combination) (µg/ml)	Ketoconazole MIC (combination) (µg/ml)	FICI	The resulting interaction type
***C. albicans* ATCC 0231**	320	2.0	160	0.06	0.53	**Additive**
***C. krusei* NRRL Y-7179**	320	2.0	10	1.0	0.53	**Additive**
***C. glabrata* ATCC 6032**	320	2.0	20	1.0	0.56	**Additive**
***C. albicans* clinical isolate**	320	2.0	160	0.06	0.53	**Additive**
***C. krusei* clinical isolate**	320	2.0	160	0.06	0.53	**Additive**
***C. glabrata* clinical isolate**	320	2.0	10	1.0	0.53	**Additive**

Table 4. Fractional inhibitory concentration index (FICI) for L2 EO.

Candida isolates	L2 MIC (alone) (µg/mL)	Ketoconazole MIC (alone) (µg/mL)	L2 MIC (combination) (µg/mL)	Ketoconazole MIC (combination) (µg/mL)	FICI	The resulting interaction type
***C. albicans* ATCC 10231**	320	4.0	320	2.0	1.5	Indifferent
***C. krusei* NRRL Y-7179**	160	1.0	10	2.0	2.06	Indifferent
***C. glabrata* ATCC 66032**	640	4.0	640	4.0	2.0	Indifferent
***C. albicans* clinical isolate**	320	4.0	160	2.0	1.0	**Additive**
***C. krusei* clinical isolate**	160	1.0	80	2.0	2.05	Indifferent
***C. glabrata* clinical isolate**	320	4.0	20	2.0	0.56	**Additive**

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Research Article

TEXTILE FINISHING WITH CHITOSAN AND SILVER NANOPARTICLES AGAINST *Escherichia coli* ATCC 8739

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Abstract: The finishing process with the antibacterial agents that protect the environment and human health is gaining importance. This study aims 1) to develop new generation antibacterial finishes using chitosan as a binder for nano-Ag coatings, 2) to determine the applicability of chitosan from shrimp and crayfish for textile production and 3) to contribute to environmentally friendly textile production. Chitosan from shrimp and crayfish wastes were used as adhesive in the binding of nanoparticles to fabric surfaces. The bonding properties of the nano-Ag particles on the fabric surfaces were investigated by Fourier transform infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), and Energy dispersive x-ray spectroscopy (EDX) analysis. The antibacterial effectiveness of fabrics against *Escherichia coli* ATCC 8739 were tested according to JIS L 1902-2015 standard. The crayfish and shrimp chitosan formed a colorless film and coated the nano-Ag particles homogeneously on the cotton fabric. Antibacterial activity values were calculated as 3.10 and 5.74 for crayfish and shrimp chitosan coated cotton fabrics and as 5.37 and 5.10 for crayfish and shrimp chitosan+nano Ag coated cotton fabrics, respectively. Chitosan nano-Ag coating which exhibited a good antibacterial activity (99.99% reduction) against *E. coli* ATCC 8739 can be used in the manufacture of garments such as medical textiles, baby clothes, and underwear. The use of chitosan as a binder can reduce the use of chemicals in textile printing and pigment dying in finishing materials, pollutant discharges and emissions from industrial sources. Also, it presents innovative solutions for the protection of human and environmental health.

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Özet: Çevreyi ve insan sağlığını koruyan antibakteriyel malzemelerle bitim işlemi son yıllarda önem kazanmaktadır. Bu çalışmanın amacı da 1) kitosanı nano-gümüş (Ag) kaplamalar için bağlayıcı olarak kullanarak yeni nesil antibakteriyel apreler geliştirmek, 2) karides ve kerevitlerden üretilen kitosanın tekstil üretimi için uygulanabilirliğini belirlemek ve 3) çevre dostu tekstil üretimine katkıda bulunmaktır. Çalışmada, karides ve kerevit atıklarından üretilen kitosan, Ag nanopartiküllerinin kumaş yüzeylerine bağlanmasında yapışkan olarak kullanılmıştır. Nano-Ag partiküllerinin kitosan aracılığıyla kumaş yüzeylerine bağlanma özellikleri Fourier dönüşümlü kızılötesi spektroskopi (FTIR), Taramalı elektron mikroskobu (SEM) ve Enerji dağılımlı x ışını (EDX) analizleri ile incelenmiştir. Kumaşların *Escherichia coli* ATCC 8739' ye karşı antibakteriyel aktiviteleri JIS L 1902-2015 standardına göre test edilmiştir. Çalışmanın sonuçları kerevit ve karides kitosanlarının renksiz bir film oluşturduğunu ve Ag nanoparçacıklarını pamuklu kumaş üzerine homojen bir şekilde kapladığını göstermiştir. Kerevit kitosanı ve karides kitosanı ile kaplı pamuklu kumaşların antibakteriyel aktivite değerleri sırasıyla, 3,10 ve 5,74 olarak hesaplanırken, kerevit kitosanı+nano-Ag ve karides kitosanı+nano-Ag ile kaplanmış pamuklu kumaşların antibakteriyel aktivite değerleri sırasıyla 5,37 ve 5,10 olarak bulundu. E. coli ATCC 8739' ye karşı iyi bir antibakteriyel aktivite sergileyen (% 99,99 azalma) kitosan+nano-Ag kaplamalar, tıbbi tekstiller, bebek kıyafetleri ve iç çamaşırları gibi giysilerin imalatında kullanılabilir. Binder olarak kitosanın kullanılması, tekstil baskısında, pigment boyamada, terbiye maddelerinde, kirletici deşarjlarında ve endüstriyel kaynaklı emisyonlarda kimyasalların kullanımını azaltabilir. Ayrıca, insan ve çevre sağlığının korunmasına yönelik yenilikçi çözümler sunar.

Introduction

Microorganisms can live in all kinds of environments including air, water and soil, and they can even survive in extreme environments such as deep-sea bottoms and volcano mouths. They are most commonly found in foods, living organisms, and clothing. In particular, cotton garments create favorable environments for bacteria with

their natural fibrous, porous and hydrophilic structure and by providing moisture, temperature and nutrient in areas that come into contact with the skin (Akaydın & Kalkancı 2014, Zhou & Kan 2014). The damage caused by microorganisms affects cotton fabrics more than other types of fabrics. Microorganisms not only cause odor formation and deterioration in the fabric, but also they cause dermal infections and allergic reactions (Tania *et al*. 2019). Colclasure *et al.* (2015) reported that clinically important coliform bacteria, such as *Escherichia coli*, can be present in significant amounts, especially on moisture-retaining fabrics at room temperatures and in dark conditions and can survive for long periods. The proliferation on the fabric surface of this pathogen bacteria, which often causes gastrointestinal infections and outbreaks of food poisoning, needs to be controlled (Pevzner 2018). Therefore, this study focused on *E. coli* ATCC 8739 and developing textile finishing agents which would give antimicrobial activity to fabrics against this pathogenic bacteria.

Polymers are materials used in various applications and are of great importance in human life. Although industrial polymers have high potential properties, they create pollution as a result of not being destroyed by natural processes (Sher *et al*. 2013). This led researchers to renewable natural resources. Chitosan is a renewable biopolymer and it is produced by deacetylation of chitin, which is a protective and supportive structure in a wide variety of living groups such as crustaceans, mollusks, insects and fungi (Abdou *et al*. 2008, Erdogan & Kaya 2016, Erdogan *et al*. 2017, Arrouze *et al*. 2017, Zhang *et al*. 2017, Song *et al*. 2018). Chitosan, which is a natural, biocompatible, biodegradable and non-toxic biopolymer, can be easily modified and functionalized thanks to its reactive hydroxyl and amino groups and it is used in diverse application areas (Anitha *et al*. 2014). Furthermore, thanks to these reagent groups, chitosan can be used in finishing and in textile dyeing (Islam & Butola 2019). The cationic structure of chitosan plays an active role by exhibiting antimicrobial, antioxidant, antitumor and anticancer properties which enables it to be used as a therapeutic and antibacterial agent in many medical applications (Anitha *et al*. 2014).

Nanoparticles are used for odor removal on textiles or controlled release of antifungals and biocides (Rivero *et al*. 2015). Nanoparticles with antimicrobial or antibacterial properties can be added to the fibers with nanotubes for long-term protection or can be added to polymers in fabric coatings. Nanosilver (nano-Ag) attracts attention with its antibacterial properties that provide an easy and lasting effect on pathogenic microorganisms like bacteria and viruses that threaten human health (Morones *et al*. 2005, Panacek *et al*. 2006). Besides, silver (Ag) nanoparticles are commercially used as a broad-spectrum antibiotic agent and exhibit high performance even at low concentrations (Govindan *et al*. 2012, Chen *et al*. 2014). Since they have a strong antibacterial effect and do not create toxic effects, Ag and Ag compounds can be used on many surfaces and areas where harmful microorganisms are abundant during production or after production (Becenen & Altun 2016). Therefore, in this study, we coated cotton fabric samples with nano-Ag particles using chitosan as a crosslinker and investigated the antibacterial activity of nano-Ag and chitosan together against *E. coli*.

Recently, a large amount of studies have been carried out to give antimicrobial properties to textile samples (Wang *et al*. 2016, Souza *et al*. 2017, Scacchetti *et al*. 2018, Fan *et al*. 2018, Rehan *et al*. 2018, Xu *et al*. 2019). In these studies, chitosan was coated on fabric surfaces via various crosslinkers or using different techniques as composite prepared with various antibacterial agents. Furthermore, in most antibacterial textile coatings, fabric samples are pretreated and modified using various chemicals before or during coating. In this study, nano-Ag particles were coated via chitosan on fabric made of 100% cellulose without pretreating and using additional chemicals. Thus, we strove to achieve in a single step both to bond nano-Ag on the fabric surface and to give antibacterial properties to the fabric, by evaluating environmental waste materials and reducing the use of chemicals. Previous studies pointed out that chitosan source affects both the physicochemical and biological properties of chitosan (Rinaudo 2006). However, there is no comparative study on how chitosan obtained from different sources affect the antibacterial performance of fabric samples. In this study, we tested the antibacterial activity of fabric samples coated with chitosan produced from two different sources (saltwater shrimp *Parapenaus longirostris* (Lucas, 1846) and freshwater crayfish *Astacus leptodactylus* (Eschscholtz, 1823)) by using the same method under controlled and standard conditions. Also, unlike other studies, we used JIS L 1902: 2015 test method, instead of the commonly used ATCC 147 and AATCC 100 tests for determining antibacterial activity of chitosan+nano-Ag coatings. Thus, we expect to obtain more realistic and accurate results with this method which reflects the real-life conditions by keeping limited the amount of nutrients in the medium.

This study aims 1) to develop new generation antibacterial finishes using chitosan as a binder for nano-Ag coatings, 2) to determine the applicability of chitosan produced from shrimp and crayfish for textile production and 3) to contribute to environmentally friendly textile production.

Materials and Methods

Materials used in the experiment

Waste shells forming the exoskeleton of shrimp and crayfish were utilized as sources of chitin and chitosan. Chitin was isolated by the chemical synthesis method from waste shells of shrimp (*Parapenaus longirostris* (Lucas, 1846)) purchased from a local market and crayfish (*Astacus leptodactylus* (Eschscholtz, 1823)) caught from Altınyazı dam lake (in Turkey). Isolated chitin were then converted to chitosan by deacetylation.

Pretreated optic white cotton calico fabric made of 100% cotton yarn was used to apply chitosan and nano-Ag particles. Nano-Ag particles (≤100 nm particle size, purity: 99.5%) were purchased from Sigma Aldrich. The nanoparticles were vortexed for 10 seconds before use to ensure homogenization of the particles.

Chitin isolation and chitosan production

Shrimp and crayfish waste shells were cleaned with water to remove impurities and then rinsed with distilled water and dried in the oven at 50 °C for 2-3 days. Chitin isolation and chitosan production from both organisms were performed following the same procedure (Fig. 1). Firstly, crushed shrimp and crayfish shells (40 g) were refluxed with 1.8 M 400 mL HCl at 60-70°C for 6 hours at 700 rpm. The acid-treated shells were then filtered through common filter paper and rinced with pure water until free of acid. The demineralized shells were then treated with 1.8 M 400 mL of NaOH at 70°C for 20 hours by stirring at 700 rpm. The mixture was then filtered again and rinsed with distilled water until the pH was neutral. Employing these two processes, minerals and proteins in waste shells were removed and chitin was obtained. Since the shells of both organisms contained a high percentage of astaxanthin pigment, the chitin obtained from these organisms were subjected to decolorization process. Decolorization was achieved by treating chitin samples with a mixture of 200 mL including chloroform, methanol and distilled water in a 1: 2: 4 ratio, respectively. This was accomplished by stirring the mixture containing chitin samples at 800 rpm for 2 hours at room temperature. Finally, the mixture was filtered and the collected chitin samples were washed with pure water and dried in the oven at 50 °C.

The dried chitin samples were refluxed with 70% NaOH solution for 3 hours at 150 °C, 800 rpm to obtain chitosan (Fig. 1). The deacetylated chitin samples were then washed continuously with pure water and the process continued until the samples were cleared of base and reached pH: 7.0. The obtained chitosan sample was placed in a petri dish and dried at 50 °C in an oven.

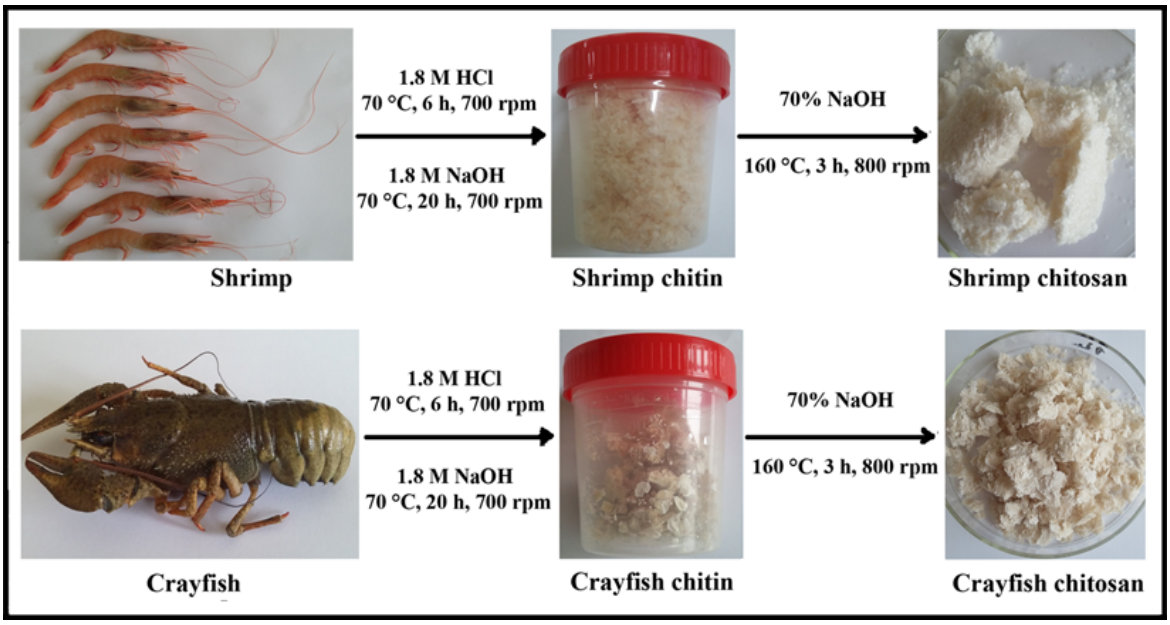

Fig. 1. Scheme illustrating the production of chitosan from shrimp and crayfish.

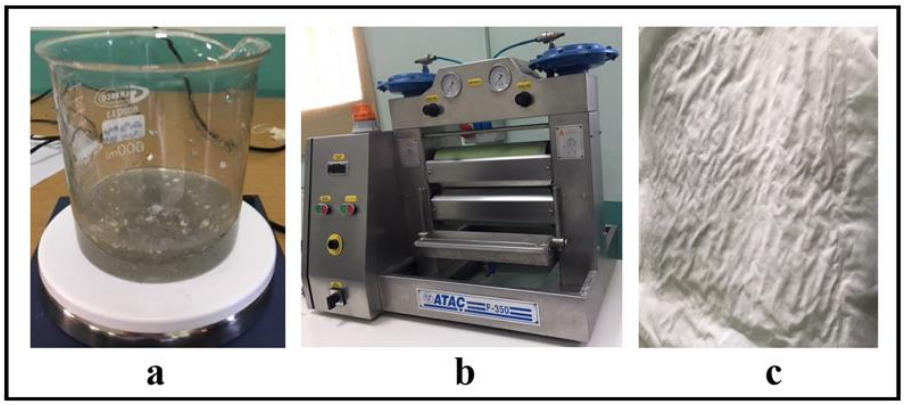

Fig. 2. Application of nano-Ag to fabric through chitosan. a) Dissolution of chitosan b) Coating machine c) Coated fabric.

Coating of cotton fabric with chitosan and nano-Ag mixture

A mixture including 10% by weight of chitosan and 1% acetic acid (CH_3COOH) was prepared using chitosan. To dissolve the chitosan, the mixture was heated at 40 °C by stirring at 400 rpm for 5 hours (Fig. 2a). Nano-Ag (0.5% by weight) was added to the prepared chitosan solutions and stirring was continued for a further 1 hour. The solutions containing chitosan and nano-Ag were impregnated with the pad-dry method on fabrics in the Ataç Brand foular machine with 100% squeezing pressure (Fig. 2b). After impregnation, the fabrics were dried at 105-110 °C and then fixed in the same machine at 120 °C (Fig. 2c).

Fourier Transform Infrared Spectroscopy (FTIR)

Chemical structure characterization was performed to confirm that the material isolated from shrimp and crayfish was chitin. Furthermore, to determine whether the chitosan+nano-Ag mixture was coated on cotton fabrics, changes in chemical bonds were determined by FTIR peaks. FTIR spectra were obtained with Perkin–Elmer ATR FTIR device in the range of 4000 to 400 cm^{-1} wavelength. Surface analyses of chitosan nano-Ag coated fabric samples were performed in Trakya University Technology Research Development Application and Research Center (TUTAGEM).

Scanning electron microscopy (SEM) and Energy-dispersive X-ray analysis (EDX)

The success of chitosan obtained from shrimp and crayfish wastes in coating nano-Ag particles on fabric samples, bonding properties between fabric and coating and the change in fabric topography was determined by SEM and EDX analyses. Whether shrimp and crayfish chitosan was successfully coated on the cotton fabric was investigated by taking surface images at different magnifications. SEM images were taken with ZEISS LEVO LS 10 and FEİ QUANTA FEG 250 SEM microscopes at 500X, 1000X and 2000X magnifications. Dispersions of nano-Ag particles on the cotton fabric surface were examined by energy dispersive x-ray analysis.

Determination of the antibacterial activity of chitosan + nano-Ag treated cotton fabrics

The antibacterial efficacy of chitosan+nano-Ag coated fabric samples against *Escherichia coli* ATCC 8739 was tested by the JIS L 1902-2015 standard test method (JIS L 1902). It is a quantitative method used to test the ability of antibacterial finished fabrics to inhibit microbial growth and kill microorganisms.

Two experimental groups as shrimp chitosan and crayfish chitosan-coated samples were formed according to chitosan source. These samples were then subdivided into 2 groups with and without nano-Ag. The antimicrobial activity of the fabric samples in each group against *E. coli* was analyzed according to the following method.

Samples (0.4 g in weight) were cut from the cotton fabric coated with chitosan and nano-Ag and placed in test tubes. Before the experiment, the fabric samples were sterilized at 121 °C for 15 minutes. As the test method, the absorption method was applied in which the bacterial suspension was directly grafted onto the samples. The fabric samples were then inoculated with the test organism of 0.2 mL at a concentration of $2.9x10^5$ CFU/mL grown in a liquid culture medium. Initial microbial concentrations were determined at time zero. Bacteria were counted according to Plate Count Method. The control and test fabric samples inoculated with *E. coli* were then incubated in closed containers at 37 ± 2 °C for 18-24 hours. After that, final bacterial concentrations were determined. The reduction of bacteria in initial concentrations and the control samples were calculated.

The bacterial growth rate for the control sample was calculated according to the formula $F = Ct-C_0$ and for the test sample it was calculated according to the formula $G = Tt-T_0$, where

A is antibacterial activity,

C_0 is the average logarithmic value of the untreated sample immediately after inoculation (0 h contact time),

Ct is the average logarithmic value of the untreated sample after 24 hours,

Tt is the average logarithmic value of the treated test sample after 24 hours and

T_0 is the average logarithmic value of the treated test specimen immediately after inoculation (0 hours).

Antibacterial activity value was calculated according to the formula A = F-G.

According to JIS L 1902 method, if $2 \leq A < 3$ then the tested sample has an effect and if $A \geq 3$, then it has a strong effect.

Results and Discussion

Chitosan as a binder

The crayfish and shrimp chitosan, used as binding agents in the coating of cotton fabrics, were treated with 1% acetic acid to form water-soluble solutions. Although there was no significant difference between the solutions, chitosan produced from shrimp wastes was easier to prepare. The shrimp chitosan produced a more homogeneous solution than crayfish chitosan and it was more easily applied to the fabric. However, the distribution of nano-Ag particles on the fabric surface was more homogeneous in the coating with crayfish chitosan compared to coatings with shrimp chitosan. Analysis results of crayfish chitosan coating were comparable to the finding of Xu *et al.* (2019) who used carboxymethyl chitosan to fix nano-Ag particles to the cotton fabric surface. Crayfish chitosan was found to be a more suitable to deposite nano-Ag particles on the fabric surface than shrimp chitosan in terms of homogeneous distribution of nanoparticles.

In coating with chitosan, the film layer on the fabric surface was colorless and did not change the color of the fabric. The film layer did not show adhesive properties after drying. Furthermore, after treatment, the chitosan solution was easily removed from the machine rollers. The chitosan used as the binding agent had no adverse effects on the binding quality of the nano-Ag particles to the fabric. These are the expected properties of fiber-bonding materials which provide the adhesion of the finishing agent to the fiber in textile finishing processes. For this reason, chitosan produced from shrimp and crayfish waste shells by the chemical method were found to be suitable for use as binder polymer in nano-Ag coatings. The use of chitosan, which is a natural and biodegradable polymer, as a binder in textile will enable to minimize the negative effects of chemicals used in finishing enterprises to the environment and contribute to environmentally friendly textile production.

FTIR analysis results

FTIR analysis was performed to characterize the chitin samples isolated from shrimp and crayfish waste shells and to confirm whether the shrimp and crayfish chitosans formed a bond between nano-Ag and cotton fabric. Chitin is available in 3 different forms as alpha, beta and gamma chitin in nature (Lavall *et al*. 2007). In the FTIR spectrum of alpha chitin, the two absorption bands at 1650 and 1620 cm^{-1} refer to the stretching of amide I, and the absorption band at 1550 cm^{-1} is attributed to amide II (N-H bending) (Focher *et al*. 1992, Lavall *et al*. 2007). The band at 650 cm^{-1} was referred to the C = O group hydrogen-bonded to N-H of the neighboring chain (Dahmane *et al*. 2016). In our study, characteristic absorption bands showing stretching of amide I of chitin were observed at 1653 and 1620 cm^{-1} for shrimp chitin and at 1655 and 1619 cm^{-1} for crayfish chitin. Another characteristic band showing amide II stretching was recorded at 1552 cm^{-1} for both shrimp and crayfish chitin (Fig. 3). This also shows that the isolated chitin is in alpha form.

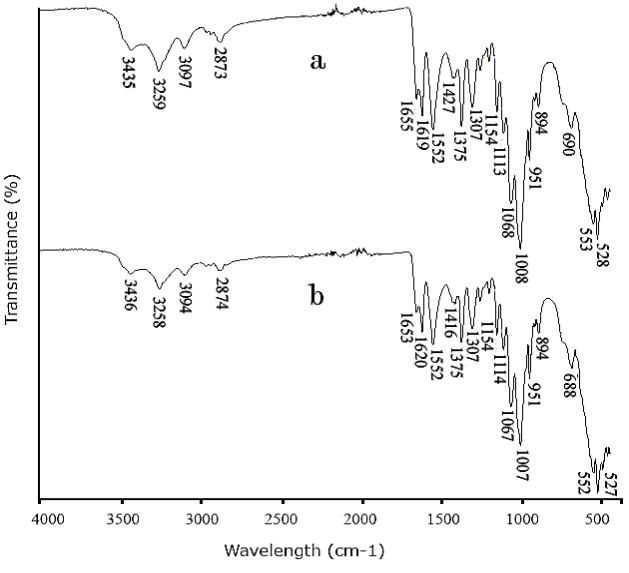

Fig. 3. FTIR spectra of isolated chitin samples. a) from crayfish, b) from shrimp.

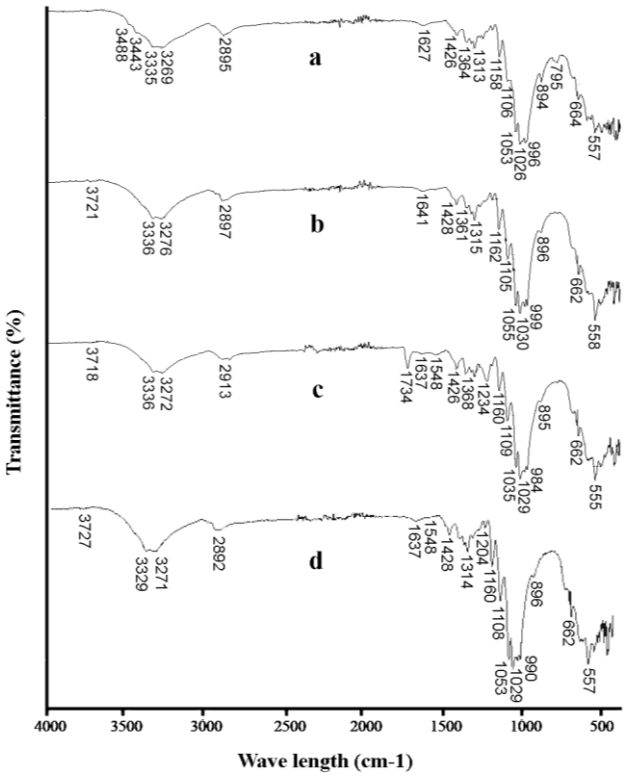

Fig. 4. FTIR images of cotton fabric coated with nano-Ag via chitosan. a) Shrimp chitosan+nano-Ag coated fabric, b) Crayfish chitosan+nano-Ag coated fabric, c) Crayfish chitosan-coated fabric and d) Shrimp chitosan-coated fabric.

The FTIR spectra of coatings using shrimp and crayfish chitosan as binders are presented in Fig. 4. Previous studies showed that the characteristic absorption bands of cellulose in the FTIR spectra of cotton fabric were observed at 1430 cm^{-1} (C-H wagging), 1364 cm^{-1} (C-H bending), 1105 cm^{-1} (C-O-C, asymmetric bridge stretching), and 1160, 1060 and 1028 cm^{-1} (C-O stretching) (Xu *et al*. 2019). These peaks were observed in our chitosan and nano-Ag coated fabric samples at 1428-1428, 1361-1368, 1105-1109, 1158-1162, 1035-1055 and 1026-1030 cm^{-1}, respectively. Mujtaba *et al.* (2016) observed a sharp peak for cellulose at 3336 and 3330 cm^{-1} attributed to the hydroxyl group and hydrogen bond, respectively. They also observed a peak at around 896 cm^{-1} correspondings to the structure of the glucose ring. These peaks were observed in our samples at 3335 and 3336 cm^{-1}, and between 894 and 896 cm^{-1}, respectively. The characteristic absorptive peaks for chitosan are I. carbonyl (C = O) band around 1650 cm^{-1} and II. amide (NH 2) band around 1590 cm^{-1} (Chen *et al*. 2014). When considering the FTIR spectra of the cotton fabrics coated with nano-Ag via chitosan, it was seen that the peak at 1650 cm^{-1} was shifted to 1633, 1641 and 1647 cm^{-1} and the peak at 1590 cm^{-1} was shifted to 1538, 1562 and 1550 cm^{-1}. Murugan *et al*. (2017) reported that the FTIR spectra of Ag/chitosan composite differed from those of chitosan. The characteristic peaks of chitosan at 1658 and 1600 cm^{-1} assigned to the stretching vibrations of amide C–O bonds sharply reduced and shifted to 1628 cm^{-1}. Another study stated that the intensity of the peak at 3434 cm^{-1} referred to the hydroxyl and primary amino groups decreased because

the amino and hydroxyl groups of chitosan chelated the nano-Ag particles (Chen *et al*. 2014).

When compared to the FTIR bands of crayfish and shrimp chitosan-coated cotton fabric (Figs 4c, 4d), changes were observed in the FTIR spectra of chitosan+nano Ag coated fabric samples (Figs 4a, 4b). As mentioned in previous studies, these shifting in the FTIR peaks indicate that the formation of coordination bonds between the amine groups of the chitosan and the nano-Ag particles (Xu *et al*. 2019), or that Ag is chelated by both amino and hydroxyl groups of the chitosan (Chen *et al*. 2014). This shows that nano-Ag is chemically bonded to cotton fabric via shrimp and crayfish chitosan.

SEM and EDX analysis results

Scanning electron microscopy was used to evaluate the presence and bonding properties of chitosan and nano-Ag particles on cotton fabric. SEM and EDX images of cotton fabrics before and after the coating showed that these fabrics were successfully coated with shrimp and crayfish chitosan and nano-Ag (Figs 5, 6). Zhou & Kan (2014) observed that while the SEM images of the fibers in the pure cotton fabric were smooth, the SEM images of the chitosan-coated cotton fibers were not smooth. Our study indicates the same results. Also, in this study, the surface of crayfish chitosan-coated fabric appeared to be rougher (Fig. 5b) than the surface of shrimp chitosan-coated fabric (Fig. 5c).

The shrimp and crayfish chitosans adhered to the cellulose fibers and formed a film by surrounding the nano-Ag particles (Figs 6a, 6d). Other studies have also confirmed that chitosan forms a film on a cotton fabric surface (Chattopadhyay & Inamdar 2013). In the coating with crayfish chitosan, nano-Ag particles exhibited a relatively homogeneous distribution (Fig. 6f), while nano-Ag particles coated on the fabric surface with shrimp chitosan appeared to aggregate (Fig. 6c). Govindan *et al.* (2012) stated that the FESEM image of chitosan–Ag nanocomposite showed that nano-Ag particles were wrapped with chitosan and agglomeration was observed. The authors also reported that agglomeration can be prevented by slowly dissolving the chitosan a good while and dispersing Ag in the chitosan with stirring for a longer time and thereby, nano-Ag particles will be embedded in a chitosan matrix.

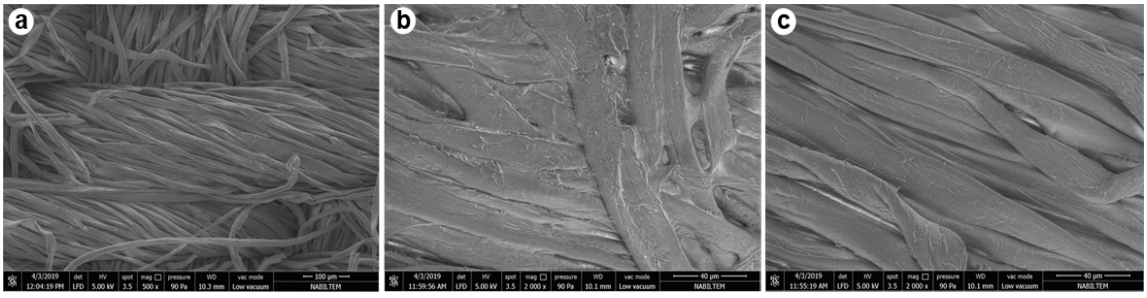

Fig. 5. SEM images of cotton fabric surfaces. a) Uncoated fabric b) Crayfish chitosan-coated fabric c) Shrimp chitosan-coated fabric.

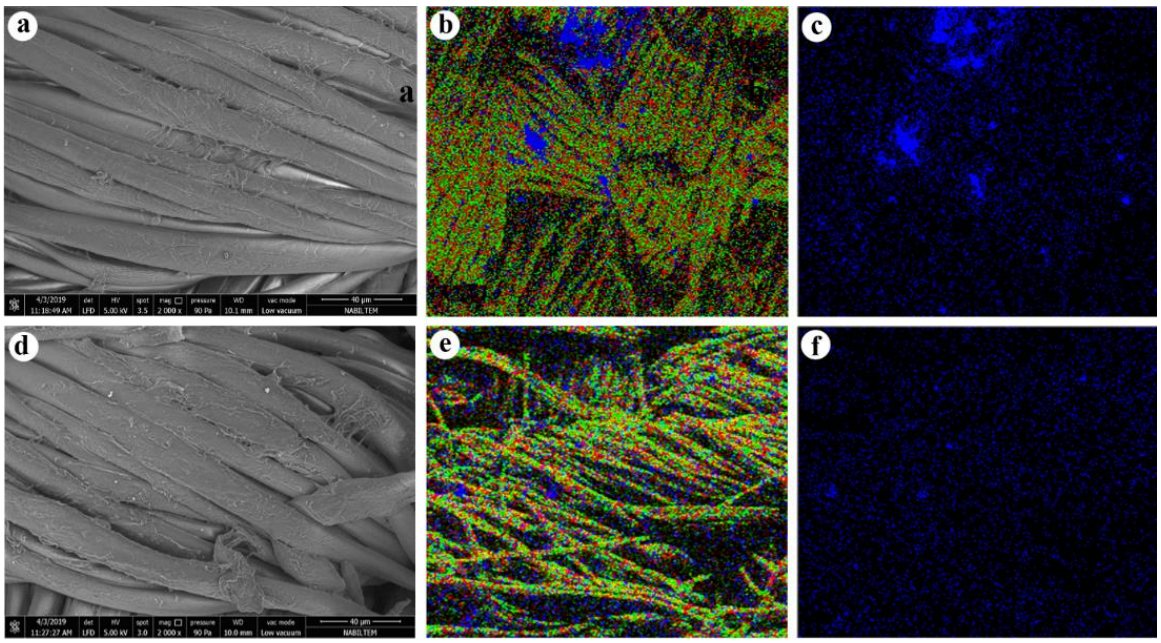

Fig. 6. SEM and EDX images of chitosan and nano-Ag coated cotton fabrics. a) SEM image of shrimp chitosan+Nano-Ag coated fabric, b and c) EDX images of shrimp chitosan+Nano-Ag coated fabric (C, O, Ag elements and Ag element only), d) SEM image of crayfish chitosan+Nano-Ag coated fabric, e) and f) EDX images of the crayfish chitosan+Nano-Ag coated fabric (C, O, Ag elements and Ag element only).

Table 1. Elemental composition of chitosan-nano Ag coatings.

	Element	Wt %	At %	K-Ratio	Z	A	F
Shrimp chitosan + Nano-Ag coating	C	46.22	53.40	0.2373	1.0076	0.5094	1.0004
	O	53.73	46.60	0.1052	0.9935	0.1970	1.0000
	Ag	0.046	0.006	0.0009	0.7667	2.7016	1.0000
	Total	100.00	100.00				
Crayfish chitosan + Nano-Ag coating	C	46.26	53.44	0.2377	1.0076	0.5096	1.0004
	O	53.67	46.54	0.1049	0.9935	0.1968	1.0000
	Ag	0.06	0.01	0.0013	0.7667	2.6772	1.0000
	Total	100.00	100.00				

EDX analysis was performed to observe the elemental distributions on the surface of cotton fabrics coated with chitosan and nano-Ag particles and to detect the presence of nano-Ag particles on the fabric surface. Previous studies reported that 3 keV peaks attributed to the Ag formation signal were observed in the EDX analysis graph of the nano-Ag deposited fabric (Gharibshahi *et al*. 2017, Tania *et al*. 2019). In this study, the presence of the peak of the Ag element in the EDX spectrum of cotton fabrics confirmed the deposition of silver nanoparticles on the fabric. Table 1 shows the distribution by weight of C, O and nano-Ag particles on the fabric surface. In previous studies, the % by weight of nano-Ag particles deposited on the fabric surface was recorded as 0.006% (Gharibshahi *et al*. 2017) and 0.10% (Arif *et al*. 2015). In our study, the weight of nano-Ag on the surface of the fabric was 0.046% in the coating with shrimp chitosan and 0.06% in the coating with crayfish chitosan. Although the amount of nano-Ag particles used in coatings is same, it appears that crayfish chitosan binds a higher amount of nano-Ag particles to the fabric in comparison to shrimp chitosan. This may be due to the acquisition of EDX images from selected regions and the aggregation of shrimp chitosan in some regions.

Antibacterial activity results of cotton fabric coated with chitosan and nano-Ag

Antibacterial activity of cotton fabric coated with chitosan and nano-Ag against *E. coli* ATCC 8739 was investigated by JIS L 1902-2015 Quantitative standard test method.

Various standard tests have been developed and used so far to determine the antibacterial efficacy of textile products. The most commonly used are qualitative tests such as ISO 20645: 2004, AATCC 147: 2004, and halo method of JIS L 1902: 2008, and quantitative tests such as AATCC 100: 2004, absorption method of ISO 20743: 2007, and absorption method of JIS L 1902: 2008 (Palamutçu *et al*. 2008, Pinho *et al*. 2011). Qualitative tests are inadequate to accurately determine the antibacterial activity of fabric samples. In quantitative methods, the reduction of bacterial growth is calculated by comparing it to a control sample and the value of the antibacterial activity can be accurately determined (Torlak 2008, Pinho *et al*. 2011). Pinho *et al*. (2011) reported that the JIS L 1902-The adsorption method gives very sensitive and accurate results. Palamutçu *et al.* (2008) stated that JIS L 1902-2002 standard is applied considering the moisture content and nutrient amount on textiles under normal clothing conditions. The authors also stated that tests are performed in a situation similar to real-life conditions and that the amount of nutrients in the medium is kept limited. Thus, the antibacterial activity of textile samples with antibacterial finishing is tested in an environment similar to real-life conditions. Table 2 presents comparative antibacterial activity values of cotton fabrics coated with nano-Ag via chitosan according to JIS L 1902-2015.

The antibacterial activity value (A) of the fabric samples covered with crayfish and shrimp chitosan were calculated as 3.10 and 5.74, respectively, considering bacterial growth rates. According to JIS L 1902-2015, if A>3, the tested sample is considered to exhibit a very good antibacterial activity (99.9% reduction). In this case, the fabrics coated with crayfish and shrimp chitosan showed high antibacterial activity against *E. coli* ATCC 8739. Besides, shrimp chitosan exhibited higher antibacterial performance than crayfish chitosan. The antibacterial activity values of cotton fabric samples coated with nano-Ag via shrimp and crayfish chitosan were calculated as 5.10 and 5.37, respectively. The reduction ratio of 99.99% in bacterial viability indicated that chitosan and nano-Ag composite coatings gave a strong antibacterial activity to cotton fabrics. The antibacterial activity values of fabrics coated with shrimp chitosan+nano-Ag and crayfish chitosan+nano-Ag were very close to each other. A significant improvement in the antibacterial activity of coatings containing chitosan+nano-Ag was observed in comparison to crayfish chitosan-coated fabric sample. Interestingly, shrimp chitosan coated fabric sample had the highest bacterial reducing rate among other fabrics.

The biocompatibility, biodegradability, non-toxicity, and antimicrobial and hypoallergenic properties of chitosan make it a suitable antibacterial agent for fabric surfaces (Zhou & Kan 2014). It has been reported that the mechanism of the antibacterial action of chitosan is to bind to the cell wall of the bacteria through its positively charged amino groups and then to adhere to DNA and prevent the proliferation of bacteria (Govindan *et al*. 2012, Zhou & Kan 2014). Results of various studies have been published on determination of antibacterial activity

Table 2. Antibacterial activities of chitosan and nano-Ag coated cotton fabrics.

Samples	Number of reproducing microorganisms (CFU/sample)		Logaritmic reduction		Growth value	Antibacterial activity value
	Contact time (0 hours)	*After incubation (24 hours)*	*Log (C0) and Log (T0)*	*Log (Ct) and Log (Tt)*	**F= Ct-C0 and *G= Tt-T0*	**A=F-G**
Uncoated cotton fabric	$7x10^4$	$6.8x10^7$	4.85	7.83	2.99	-
Shrimp chitosan and nano-Ag coated fabric	$5.14x10^4$	$4x10^2$	4.71	2.60	-2.11	A=5.10 A>3 %99.99
Crayfish chitosan and nano-Ag coated fabric	$4.84x10^4$	$2x10^2$	4.68	2.30	-2.38	A=5.37 A>3 %99.99
Shrimp Chitosan coated fabric	$4.1x10^4$	$2x10^2$	4.61	2.30	-2.31	A=5.74 A>3 %99.99
Crayfish Chitosan coated fabric	$7.8x10^2$	$6x10^4$	4.89	4.78	-0.11	A=3.10 A>3 %99.9

$F= Ct-C_0$: Growth value of uncoated fabric, *$G= Tt-T_0$:* Growth value of coated fabric

of chitosan against various bacterial species (Jiang *et al*. 2010, Velmurugan *et al*. 2014, Tania *et al*. 2019). A study reported that chitosan-coated cotton fabric samples effectively inhibited the growth of *Staphylococcus aureus* and *Klebsiella pneumoniae* (Zhou & Kan 2014). Şahan & Demir (2016) reported that both chitosan and nano chitosan showed good antibacterial activity against *S. aureus*, *E. coli* and, *K. pneumoniae*. The results of this study indicated that crayfish chitosan showed high antibacterial effeciency against *E.coli*. Chitosan source affects the physico-chemical and biological properties of chitosan (Kumirska *et al*. 2011). The results of this study confirmed that the source of chitosan affects the biological properties of chitosan. Shrimp chitosan showed higher antibacterial performance than crayfish chitosan.

Nano-Ag particles have been widely used in the development of antibacterial textiles as effective antibacterial agents in recent years. Previous studies emphasized that Ag showed high biocompatibility and low toxicity with human cells in both ionic and colloidal forms (Marambio-Jones & Hoek 2010). Since nano-Ag particles have a large surface area compared to their volume, they provide better contact with microorganisms and thus show good antibacterial properties (Velmurugan *et al*. 2014). A study reported that Nano-Ag particle coated cotton fabric has an excellent bacterial reduction for *S. aureus* (95%) and *E. coli* (92%) (Tania *et al*. 2019). Another study reported that the bacterial reduction rates of nano-Ag particle coated polyester fabrics were 99.7% for *E. coli* and 99.8% for *S. aureus* (Jiang *et al*. 2010). Both studies agree that nano-Ag particles exhibit excellent bacterial reduction and this effect related to the amount of nano-Ag particles deposited on the fabric surface. Microbial growth reduction values of nano-Ag -containing composites for *S. aureus* and *Pseudomonas aeruginosa* were measured between 3.3-7.0 log CFU by JIS L 1902 method (Wiegand *et al*. 2015).

Chattopadhyay & Inamdar (2013) reported that nano chitosan exhibited better antibacterial activity when treated with nano-Ag. Chen *et al*. (2014) reported that Ag/chitosan composites exhibit higher antibacterial activity against both the gram-positive bacteria *S. aureus* and *Bacillus subtilis* and the gram-negative bacteria *E. coli* and *Salmonella choleraesuis* than chitosan. The authors concluded that as well as the homogenously and well distribution of nano-Ag particles in the chitosan matrix, also synergistic effects of chitosan and nano-Ag particles caused Ag/chitosan composites to exhibit higher antibacterial activity. Arif *et al*. (2015) measured the antibacterial activity of fabric samples coated with chitosan+nano-Ag particles against *S. aureus* and *E. coli* and they found the bacterial reduction rates to be around 98-99%. The authors stated that there was only a slight reduction in antibacterial activity even after 20 washes. Xu *et al*. (2019) coated carboxymethyl chitosan/Ag nanoparticle colloidal solution on a cotton fiber surface by the pad-dry-cure method using carboxymethyl chitosan as a binder. The authors stated that this coating was successfully coated on the fabric surface and imparted to the fabric significant antibacterial activity against *S. aureus* and *E. coli*. They discovered that even after 50 washes, the fabric retains its antibacterial activity. The results of relevant studies have shown that the application of chitosan together with Ag particles increases the antibacterial effect, and some results of our study are consistent with these studies. Crayfish chitosan+nano-Ag composite (A= 5.37) showed a higher antibacterial effect than crayfish chitosan (3.10). On the other hand, shrimp chitosan (A= 5.74) exhibited higher antibacterial activity then shrimp chitosan+nano-Ag composite (A= 5.10).

Evaluation of the chitosan and nano-Ag coatings in terms of human and environmental health

It is expected that antibacterial textile coatings are safe for human health and environmentally friendly. Chitosan is a biocompatible, biodegradable and non-toxic

glycopolymer of biological origin. These properties of chitosan, which are used for treatment in many medical applications, have been emphasized in many studies (Li *et al.* 2016, Liang *et al*. 2018, Zhao *et al*. 2018). Chitosan is degradable within the body and it is safe and non-toxic (Dutta *et al.* 2004). In vertebrates, it is known that chitosan is mainly degraded by lysozyme and certain bacterial enzymes in the large intestine (Dash *et al.* 2011). It can also be degraded by many microorganisms in nature. Nano-Ag particles are also not prohibited and are one of the most suitable and commercially distributed nanomaterials in the world (Korani *et al.* 2015). Nano-Ag particles are widely used in medical and functional textiles, wound dressings, medical devices implanted for a long time, dental materials, water disinfectants, room sprays, laundry powders and deodorants due to their antibacterial and deodorizing properties (SCENIHR 2014, Korani *et al.* 2015, Burdusel *et al.* 2018). However, researches on the effects of nano-Ag particles on human health are limited (Korani *et al*. 2015). In vitro studies revealed that nano-Ag particles may show cytotoxic and genotoxic effects (SCENIHR 2014). Hartemann *et al.* (2015) reported that in vitro studies showed that nanosilver induced cytokine production in macrophages. Since there are few studies on the genotoxicity of nano-Ag particles in vivo, it has been reported that the results of the present studies can not confirm the positive or negative effects of nano-Ag particles (SCENIHR 2014). The SCENIHR report states that the results of the studies are contradictory and some of them said that nano-Ag showed high cytotoxicity at doses between 2-5 μg/mL, while the others showed almost no cytotoxicity at doses up to 100 μg/mL (SCENIHR 2014). Korani *et al.* (2013) showed that nano-Ag particles cause histopathological abnormalities in spleen, liver, and skin in animal experiments. In another study, colloidal nano-Ag particles were reported to be capable of producing a dose-dependent toxic response in several organs (Korani *et al*. 2015). However, many studies point out that there is a lack of consistent and reliable data on the toxicity and biological behavior of nano-Ag particles in both in vitro and in vivo toxicity studies and further studies are needed to produce meaningful results (Korani *et al.* 2015, Hartemann *et al.* 2015, Burdusel *et al*. 2018). Researchers concluded that many parameters including deposition rates, particle size, surface area, dose taken, interaction with biological macromolecules, dispersion ratio, concentration, surface charge, morphology, surface oxidation, and conversion under biological conditions affect the toxicity of nano-Ag particles (SCENIHR 2014, Hartemann *et al.* 2015, Korani *et al.* 2015, Burdusel *et al.* 2018). Researchers emphasize the necessity of long-term studies with wide-range doses and different particle sizes to accurately determine the effects of nano-Ag particles on human health, (SCENIHR 2014, Korani *et al.* 2015).

When the published data on the subject is examined, it is seen that the findings related to the genotoxic and cytotoxic effects of nano-Ag particles are generally related to the intake of these nanomaterials as food supplements or drugs. However, in antibacterial textile products, nano-Ag is used on the fabric surface and it is only in contact with the skin. As in this study, it is generally used in low doses. In this study, SEM analyzes of chitosan and nano-Ag coated fabrics revealed that chitosan forms a film that encapsulates nano-Ag particles and fixes them to the fabric. The nanoparticles are embedded in the chitosan matrix. This is also noted in previous studies (Govindan *et al.* 2012, Chattopadhyay & Inamdar 2013). Chitosan is already a natural biomaterial and it prevents direct contact of nano-Ag on the fabric surface with the skin. Thus, it prevents possible toxic effects by preventing or limiting the penetration of nano-Ag particles into the body. Edward-Jones (2009) stated that although, Ag element has been used intensively in the treatment of burns for 50 years, there are limited reported cases of Ag toxicity. The author states that Ag poisoning is dependent on Ag levels absorbed into the body in time and the amount absorbed by intact skin are lower than that absorbed by open wounds. Furthermore, SCENIHR (2014) reports that the uptake of nanomaterials via the skin is generally very low. Considering all these data, it can be concluded that the chitosan/nano-Ag coatings are safe for human health and environmentally friendly because they minimize the use of chemicals.

Little is known about the environmental impacts and hazards of nano-Ag particles in aquatic systems and it is not possible to draw general conclusions (SCENIHR 2014, Korani *et al*. 2015). Hartemann *et al.* (2015) state that nano-Ag particles released into the environment have a transformation such as aggregation, agglomeration, dissolution or silver chloride and silver sulfide formation. The author says that the chemical species of the transformed nano-Ag determines the bioavailability and toxicity of Ag in nature. He also stated that the effects of nano-Ag particles on soil can be vary depending on both nanoparticle and soil properties. As for water sources, Ag can be used to control the bacteriological quality of drinking water. The WHO (2017) report states that although there is insufficient data to obtain a health-based guide for Ag in drinking water, Ag levels up to 0.1 mg/L can be tolerated.

Conclusion

Chitosan formed a colorless film and a matrix enabling nano-Ag particles to deposit homogeneously on the fabric surface. Chitosan source was effective in the quality of chitosan coatings. Crayfish chitosan may be more suitable for more homogeneous and thinner coatings. These properties are sought after in textiles and indicate that chitosan is appropriate to be used as a finishing agent in textiles. Also, the method used to coat fabrics is applicable to the long length and it is a useful method. It can be applied to the industry due to its ease of use and low cost.

Since the JIS L 1902 method allows comparative evaluation of the antibacterial activity of treated and untreated fabrics, it increases the reliability of the results and is considered to be a suitable method for testing

antibacterial activity. A very strong antibacterial effect (99.99% reduction) was obtained against *E. coli* bacteria on pre-treated cotton fabric surfaces with the effect of both nano-Ag particles and chitosan. Chitosan nano-Ag coating which exhibited a good antibacterial activity can be used in the manufacture of garments such as medical textiles, baby clothes, and underwear because of its sterility, due to its biocompatible, biodegradable, non-toxic and sterilizable properties.

Chitosan nano-Ag coating used as finishing contributes to the protection of human health by reducing the use of antibiotics. The evaluation of shrimp and crayfish wastes as chitosan also contributes to the protection of the environment. Besides, the chemical ratio can be reduced by the use of chitosan instead of binder, which is commonly used in textile printing, pigment dyeing in finishing materials. Purification processes can be reduced and thus cost is reduced. By structuring a clean production approach in textile enterprises and sustainable use of natural resources, it is possible to prevent or reduce pollutant discharges and emissions from industrial sources. Thus, this approach offers innovative solutions for the protection of human and environmental health.

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Research Article

Callistosporium SINGER, A NEW GENUS RECORD FOR TURKISH MYCOBIOTA

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Molecular phylogeny
New record

Abstract: This study aims to describe and introduce a new record for the Turkish mycobiota. Based on the similar macro- and micromorphology, and high nuclear ribosomal large subunit sequence similarity, the mushroom was identified as *Callistosporium olivascens* (Boud.) Bon. According to the literature research, we found out that this finding is the first record of the genus Callistosporium in Turkey.

Özet: Bu çalışmanın amacı Türkiye mikobiyotası için yeni bir kaydı tanıtmaktır. Benzer makro- ve mikromorfoloji ve yüksek çekirdek ribozomal büyük alt ünite dizi benzerliğine bakılarak bu mantar *Callistosporium olivascens* (Boud.) Bon. olarak teşhis edildi. Literatür araştırmalarına göre bu bulgu cinsin Türkiye'deki ilk kaydıdır.

Introduction

Callistosporium Singer is a small genus of the family *Tricholomataceae* within the order *Agaricales* (*Basidiomycota*). According to Index fungorum, sixteen confirmed species (*C. amazonicum* Singer, *C. chrysophorum* Singer, *C. elegans* Desjardin & B.A. Perry, *C. olivascens* (Boud.) Bon, *C. luteo-olivaceum* (Berk. & M.A. Curtis) Singer, *C. foetens* E. Ludw., *C. galerinoides* Singer, *C. heimii* (Singer) Singer, *C. krambrukum* Grgur., *C. marginatum* (Peck) H.E. Bigelow, *C. palmarum* (Murrill) Singer, *C. pinicola* Arnolds, *C. purpureomarginatum* Fatto & Bessette, *C. terrigenum* Singer, *C. vinosobrunneum* Desjardin & Hemmes, and *C. xerampelinum* Pegler) currently exist in the genus. Its members are characterized by collybioid or mycenoid basidiomata, convex to plane, umbilicate or umbonate, thin and firm, hygrophanous, yellow, olivaceous, brown, sometimes dark vinaceous brown pileus, frequently farinaceous odor; thick and subdistant, adnexed to adnate, dark vinaceous brown to yellow and waxy lamellae, dark vinaceous brown, yellowish to greenish, central and tough stipe, white spore print, hyaline, four- spored, sometimes pigmented basidia, subglobose to ellipsoid, hyaline or intracellular pigmented basidiospores, generally lacking of cystidia and clamp connections (Singer 1944, Kühner & Romagnesi 1954, Lennox 1979, Bon 1984, Moser 1986, Contu 1993, Bas *et al*. 1996, Jančovičová *et al*. 2016).

According to literature on Turkish mycobiota (Sesli & Denchev 2008, Uzun *et al*. 2014, Akata & Doğan 2015, Sesli *et al*. 2016, Acar *et al*. 2017, Öztürk *et al*. 2017, Akata *et al*. 2018, 2019, Altuntaş *et al*. 2019, Acar *et al*. 2020), approximately 2500 macrofungi species have thus far been registered from Turkey but there exists no report related to the genus *Callistosporium* Singer in the country. This study aims to introduce this genus in Turkey and to make a new contribution to Turkish mycobiota.

Materials and Methods

Basidiomata of the study were collected from Turkey, Ankara University Beşevler 10.Yıl campus (date: 20.09.2018). Color, odor, surface structure and mycorrhizal relationships of fruiting bodies were noted in the field. Freehand sections were obtained from pileus, stipe, and lamellae to examine the microscopic structures. Sections were mounted in both distilled water and concentrated ammonia. They were then stained with Congo red and examined using the Euromex Oxion Trinocular microscope. 100X magnification rates were used for microscopic structures and at least 20 measurements were performed. Identification was made using morphological and molecular methods (Singer 1944, 1946, Kühner & Romagnesi 1954, Lennox 1979, Bon 1984, 1991, Moser 1986, Contu 1993, *Bas et al.* 1996, Jančovičová *et al.* 2016, Pancorbo *et al.*2016,

Conca *et al.* 2017). The identified samples are deposited in Ankara University herbarium (ANK).

Molecular characterization

DNA Isolation

The genomic DNA of the specimens ANK Akata & Altuntas was isolated from the fruit bodies according to the CTAB method (Doyle & Doyle 1987). NanoDrop One© Microvolume UV-Vis Spectrophotometer (Thermofisher) was used to measure DNA concentration and purity.

PCR Amplification and Sequencing

The nuclear ribosomal large subunit (nrLSU) region of the rDNA was PCR amplified using the universal LR0R and LR5 oligonucleotide primers (Stielow *et al*. 2015). PCR was conducted in a reaction volume of 25µmL. The final concentrations of the PCR ingredients were adjusted as follows: 1 × Taq DNA polymerase buffer, 1 unit of Taq DNA polymerase (Fermentas), 0.4 mM dNTPs, 2.5 mM MgCl2, and 10 pmol of both LR0R and LR5 primers. PCR was carried out in a Thermal Cycler (Applied Biosystems MiniAmp Plus) with the following thermal cycling conditions: initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C for 30s, 55°C for 30 s, and 72°C for 90 s, and a final extension step of 7 min at 72°C. The PCR amplicon was electrophoretically separated in 1% agarose gel containing the intercalating dye ethidium bromide, and the amplicon size was determined using a DNA marker (GeneRuler 100 bp Plus DNA Ladder, Thermofisher). The amplicon was sequenced bidirectionally using the LR0R and LR5 primers and the standard Sanger dideoxy chain termination method at the laboratory of Macrogen Europe (Amsterdam, The Netherlands).

Sequence Analysis

The nrLSU gene sequences of some relevant fungal species were obtained from GenBank and used for the phylogenetic analysis of the specimen Ank Akata & Altuntas 172. While the nrLSU sequences of the genera *Callistosporium* Singer, *Singerocybe* Harmaja, *Tricholoma* (Fr.) Staude and *Lepista* (Fr.) W.G. Sm. as some of the well-known genera of *Tricholomataceae* R. Heim ex Pouzar were selected as in-group sequences, the nrLSU sequences of *Agaricus campestris* L. and *Marasmius oreades* (Bolton) Fr. were selected as the out-group sequences. The sequences were assembled using Geneious Prime 2019.1.3 software (Biomatters Ltd) and used for the sequence identity analysis with Basic Local Alignment Search Tool (BLAST). The DNA sequences were then aligned using the CLUSTALW. Molecular phylogenetic analysis was conducted using the neighbor-joining method based on the K2 + G substitution model. MEGAX software was used for constructing the phylogenetic tree by applying one thousand bootstrap replicates (Kumar *et al*. 2018, Felsenstein 1985).

Results

Family *Tricholomataceae* R. Heim ex Pouzar

Genus *Callistosporium* Singer

Callistosporium olivascens (Boud.) Bon, 1976. (Figs 1, 2).

Syn.: *Collybia aerina* Quél. 1884, Assoc. Franç. Avancem. Sci., Congr. Rouen 12: 498 (1884).

= *Tricholoma olivascens* Boud., Bull. Soc. Mycol. Fr. 33(1): 7 (1917).

= *C. olivascens* var. *aerinum* (Quél.) Bon, Docums Mycol. 6(23): 286 (1976).

Macroscopic and microscopic features

Basidiomata clustered to solitary. **Pileus** 20-50 mm, hemispherical when young, later campanulate, convex to almost plane or funnel-shaped depending on weather conditions, with a wide umbo. Margin straight to wavy, entire, translucently striate; surface smooth, velutinous to apparently glabrous; mixture of brown, yellow and green pigments and hygrophanous. **Lamellae** sparse, L = 25-35, l = 1-3, emarginate, pale yellowish to beige when young, then rusty yellow or olive-yellow**. Stipe** 20-50 × 2-4 mm, generally central, cylindric, fused into a cluster, mostly curved, longitudinally compressed, fistulose, brown, yellowish-brown and olive-brown, sometimes minutely floccose or finely longitudinally fibrillose. **Context** 1.5-2 mm thick, olive-brown or yellowish. **Taste** mild, smell like beeswax. **Basidiospores** 7.5-9.5 × 5-6.5 µm, ellipsoid with small but distinct hilar appendage, hyaline, smooth, thin-walled with various content. **Basidia** 35-40 × 6-7 µm, clavate to cylindrical, 4-spored, clavate, thin-walled, hyaline with globose droplets. **Hymenial cells** are clavate, narrowly utriform and cylindrical with obtuse apex**. Cheilocystidia** and **pleurocystidia** not seen. **Pileipellis** a cutis, about 30-70 mm deep, made up of cylindrical, smooth or incrusted, thin- to thick-walled, 3-8 mm wide hyphae; Terminal cells cylindrical, narrowly cylindrical, clavate, narrowly clavate or narrowly lageniform. **Stipitipellis** a cutis of cylindrical, smooth or slightly incrusted, thin- to slightly thick-walled, up to 9 mm wide hyphae. **Clamp-connections** absent in all tissues.

Specimen examined: TURKEY-Ankara: Ankara University Beşevler 10.Yıl campus, under deodar cedar (*Cedrus deodara* (Roxb. ex D.Don) G.Don), 867 m, 39°56'04" N, 32°50'00" E, 20.09.2018, ANK Akata & Altuntas 172.

Molecular Phylogeny of the Specimen

As a result of the phylogenetic analysis, four distinct clades were revealed along with an out-group. The clade 1 contained *Callistosporium* species and the specimen Ank Akata & Altuntas 172. The Clades 2, 3 and 4 included species from the genera *Singerocybe*,

Fig. 1. a-c. Basidiomata of *Callistosporium olivascens* (photographed by Ilgaz Akata).

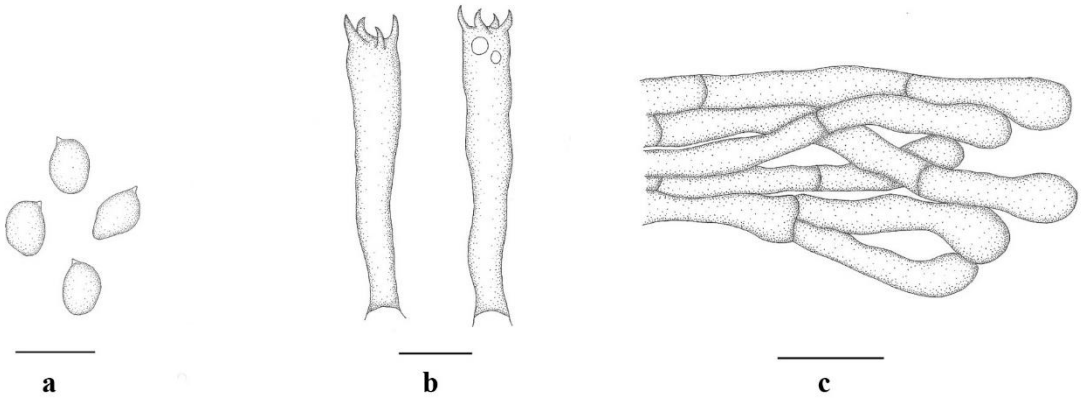

Fig. 2. *Callistosporium olivascens* (illustrated by Deniz Altuntaş) a. basidiospores, b. basidia, c. pileipellis (bar: 10 µm).

Tricholoma and *Lepista*, respectively. On the other hand, *A. campestris* and *M. oreades* were branched far from the rest of the fungi species and formed an out-group as anticipated. The BLAST analysis carried out with the nrLSU sequences of Ank Akata & Altuntas 172 provided evidence for the 99.89% similarity of this new record with the two separately collected *C. olivascens* specimens. The phylogenetic analyses conducted based on the nrLSU sequences of this specimen further supported the close identity relationship of the specimen with *C. olivascens* with a percent bootstrap value of 98 (Fig. 3).

Discussion

Although the genus *Callistosporium* includes sixteen confirmed species, the most known European members are *C. olivascens* (Boud.) Bon, *C. luteo-olivaceum* (Berk. & M. A. Curtis) Singer and *C. pinicola* Arnolds (Jančovičová *et al.* 2016). These species may be confused in the field due to their collybioid habit but *C. olivascens* can be distinguished from the latter species by its different morphology and ecology. *Callistosporium olivascens* was described in this study with 20-50 mm, hemispherical, campanulate, convex to almost plane or funnel-shaped pileus; emarginate, pale yellowish to beige, rusty yellow or olive-yellow lamellae, 7.5-9.5 × 5-6 µm and ellipsoid basidiospores. *Callistosporium luteo-olivaceum* and *C. pinicola* have narrower pileus and spores. While *C.* luteo-*olivaceum* has up to 35 mm broad pileus and (4.2-)4.7-5.6(-6) × (3-)3.3-4(-4.2) µm, basidiospores, *C. pinicola* up to 32 mm broad pileus and (2.5-)3-4(-4.5) × 2-3(-3.5) µm spores (Antonín *et al.* 2009, Jančovičová *et al.* 2016).

Since the morphological data is not always adequate for the precise identification of fungal species, the sequence data from the conserved DNA regions such as ITS, nrSSU and nrLSU has been employed as a convenient tool in taxonomic studies in the last three decades (Raja *et al.* 2017). Furthermore, nrLSU is one of the most common DNA barcoding markers and thus confers important information for molecular phylogenetic studies. Therefore, we used nrLSU region for the molecular identification of the specimens Ank Akata & Altuntas 172. The phylogenetic analysis conducted based on the nrLSU regions revealed almost 100% genetic similarity between the *C. olivascens* (GenBank ID: MK277665) and the new record (GenBank ID: MN486509 for Ank Akata & Altuntas 172) (Fig. 3).

The molecular phylogeny of *C. olivascens* demonstrated herein, points out its significant distinction from the other species of the genus *Callistosporium* as *C. olivascens* clusters in a separate branch within the clade 1 (Fig. 3). Based on this finding, it is plausible to state that the taxonomic revision of this species is likely to be addressed in the future.

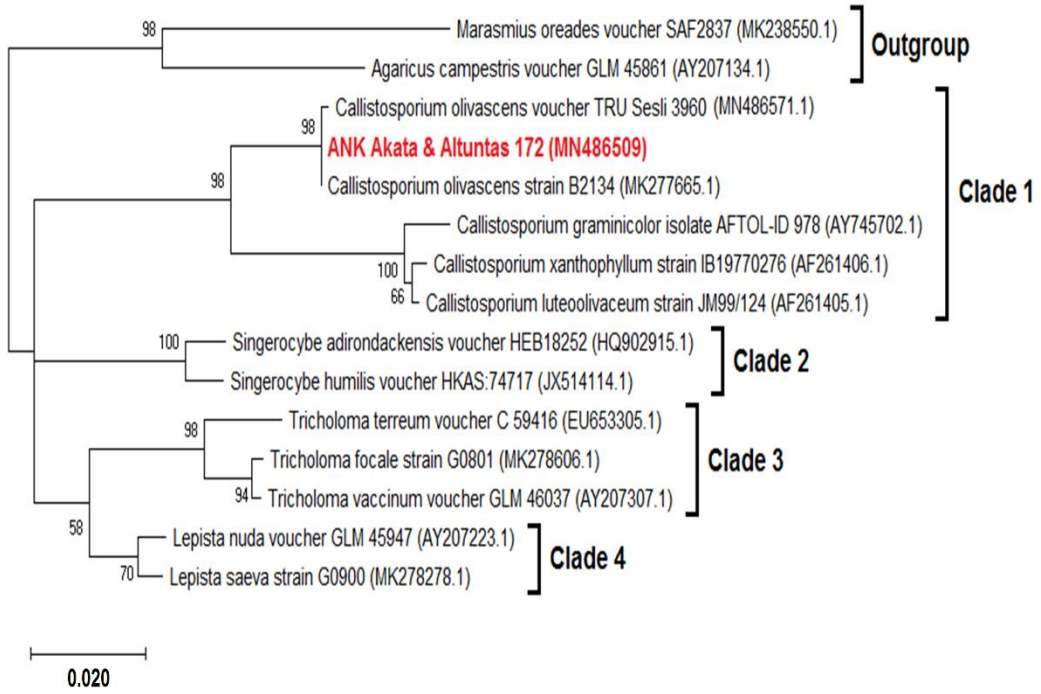

Fig. 3. The neighbor-joining tree demonstrating the phylogenetic relationships of 15 fungi inferred from the nrLSU region. Percentage bootstrap values obtained from 1000 replicates were given next to the branches. All the sequences used in the phylogenetic analysis were obtained from GenBank except for Ank Akata & Altunta 172. *Agaricus campestris* and *Marasmius oreades* were used as the outgroup samples. Accession numbers are given in parentheses. The scale bar at the lower left represents a genetic distance of 0.02

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Editor-in-Chief note: One of the author in this paper, Ilgaz Akata, is a member of Editorial Board of Trakya University Journal of Natural Sciences. However, he was't involved in the decision process during manucript evaluation.

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Research Article

IMMOBILIZATION OF *Bacillus subtilis* E6-5 PROTEASE AND COMMERCIAL PROTEASE IN NANOFIBRILS CONTAINING DIFFERENT AMINO ACIDS

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Abstract: In this study, polyamide 6 polymer surfaces that have a high surface area were produced by electrospinning method with the participation of Glycine, Tyrosine and Glutamic acid amino acids, and lyophilized *Bacillus subtilis* E6-5 protease and commercial protease enzymes were immobilized on nanofibrils. Enzyme reusability were investigated. The immobilization efficiencies of the enzymes were approximately between 50-55 %. In studies with lyophilized *Bacillus* protease, glutaraldehyde activated PA6 nanofibrils and glutaraldehyde unactivated PA6 nanofibrils were found to be more immobilized in the presence of Glutamic acid. Although the lyophilized protease enzyme immobilized on non-glutaraldehyde activated and activated surfaces has been used 4 times, the best functional stability has been achieved with 2 times use. In pure PA6/Glutamic acid nanofibrils, the immobilization yield of the two times used enzymes was found to be 38 %. In glutaraldehyde-activated PA6 nanofibrils, the PA6/Glutamic acid nanofibril surfaces were found to have 65 % immobilization yield of the two repetitive used enzymes. The enzyme immobilization efficiency has been doubled by glutaraldehyde activation of the nanofibrils. In studies with commercial protease, the most functional stability was obtained for 3 repeated uses, although the enzyme was used 6 times on the non-glutaraldehyde activated nanofibril surfaces. The most successful immobilization was found in 58 % of PA6 nanofibrils. In glutaraldehyde-activated PA6 nanofibrils, the enzyme was found to be used 6 times, but the functional stability was maintained as much as 4 times of repeated use.

Özet: Bu çalışmada, elektrospin yöntemiyle yüksek yüzey alanına sahip, glisin, tirozin ve glutamik asit aminoasitleri ile oluşturulmuş poliamid 6 polimer yüzeyler üretilmiş ve liyofilize *Bacillus subtilis* E6-5 proteaz ve ticari proteaz enzimleri nanofibriller üzerinde immobilize edilmiştir. Enzimlerin yeniden kullanılabilirliği araştırıldı. Enzimlerin immobilizasyon verimlilikleri yaklaşık olarak % 50-55 arasındaydı. Liyofilize *Bacillus* proteazı ile yapılan çalışmalarda glutaraldehitle aktifleştirilmiş PA6 nanolifler ve glutaraldehitle aktifleştirilmeyen PA6 nanoliflerde glutamik asit aminoasidi varlığında immobilizasyonun daha başarılı olduğu saptanmıştır. Glutaraldehit ile aktifleştirilmemiş ve aktifleştirilmiş yüzeylerde immobilize edilen liyofilize proteaz enziminin 4 kez kullanımı olmasına rağmen, en iyi işlevsel stabilite 2 kez kullanım ile elde edilmiştir. Saf PA6/glutamik asit nanoliflerinde iki tekrarlı kullanım sonucu enzimin immobilizasyon verimi % 38 olarak bulunmuştur. Glutaraldehitle aktifleştirilmiş PA6 nanoliflerde de PA6/glutamik asit nanolif yüzeyleri iki tekrarlı kullanım sonucu enzimin immobilizasyon verimi % 65 olarak bulunmuştur. Nanoliflerin glutaraldehitle aktifleştirmesi sonucu enzim immobilizasyon verimi iki kat arttırılmıştır. Ticari proteaz ile yapılan çalışmalarda ise glutaraldehitle aktifleştirilmemiş nanolif yüzeylerde enzimin 6 kez kullanımı olmasına rağmen en işlevsel stabilite 3 tekrarlı kullanımda elde edilmiştir. En başarılı immobilizasyon verimi PA6 nanoliflerde % 58 olarak bulunmuştur. Glutaraldehitle aktifleştirilmiş PA6 nanoliflerde de enzim 6 kez kullanım bulmuş fakat işlevsel stabilite 4 tekrarlı kullanıma kadar korunmuştur.

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Introduction

The use of enzymes in a soluble or free form must be considered as wasteful because the enzyme generally cannot be recovered at the end of the reaction. Utilization of enzymatic reactions for the industrial processes have been restricted due to relatively high enzyme costs. Most of the industrial enzymatic processes are performed batchwise using soluble enzymes. In order to re-use enzymes after reactions and to increase their stability, their immobilizations using an insoluble carrier have been broadly studied (Chaplin 1990). Enzyme immobilization

can currently be carried out in different ways such as adsorption, covalent binding, entrapment and encapsulation (Taher 2011). Physical adsorption has been selected among these immobilization methods by many researchers considering its easy applicability, ability to maintain the enzymatic activity and the lack of expensive costs and toxic waste products (Al-Zuhair 2007).

A large variety of nano-structured polymers such as nanofibrils, nanoparticles, silica, and nanotubes have been used as solid supports. Electrospun nanofibrils have great potential for enzyme immobilization (Wang 2009). Nanofibrils have a large number of applications which blended wound healing, energy generation and storage, filtration and enzyme immobilization. Polyamide 6 nanofibrils (PA6 NFs) are practical materials for enzyme immobilization studies as they are easily available (Chen 2005).

In this study, the immobilizations of the previously isolated novel *Bacillus subtilis* protease and commercial protease (ORBA Biochemistry, Istanbul) in nanofibers containing different amino acids were investigated. The reusability of the nanofiber was also tested.

Materials and Methods

Bacterial Protease Production

Production of *Bacillus subtilis* protease was performed in flasks containing a medium including) glucose 0.1, peptone 1, yeast extract 0.02, $MgSO_4$ 0.01, $CaCl_2$ 0.01 and K_2HPO_4 0.05 (pH 7.0) (Qadar *et al.* 2009). Glucose was sterilized and added to the flasks separately. Overnight *B. subtilis* cultures in Nutrient Broth medium (0.8 % w/v) with an optical density of 0.3 at 600 nm were inoculated at 1 % in enzyme production media (250 mL in 1000 mL Erlenmeyer flasks) and incubated at 37°C for 48 h in a shaking incubator at 150 rpm. After incubation, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for lyophilization.

Assay of protease activity

Total protease activity was measured using a casein substrate by a modification of the Anson Method (Keay and Wildi 1970). 1 mL aliquot of the culture supernatant was mixed with 1 mL 0.05 M phosphate buffer -0.1 M NaOH (pH 7,0 adjusted with phosphoric acid) containing 2 % casein and incubated for 10 min at 37°C. The reaction was stopped by adding 2 mL 0.4 M Trichloroacetic acid. After 30 min stands at room temperature, the precipitate was removed by centrifugation (6000 rpm, 10 min) and the optical density of the assays was measured at 660 nm. A standard curve was generated using solutions of 0-60 µg/mL Tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/mL Tyrosine under the experimental conditions used.

Lyophilization of Bacillus subtilis E6-5 protease

The crude enzyme extract obtained as a result of 1000 mL production in the enzyme production medium was kept at -20ºC overnight. The frozen samples were lyophilized (CHRIST Alpha 2-4 LD plus). The lyophilization process was carried out at -55ºC and the enzyme sample was powdered. Powder samples were dissolved in pure water (20 mL) to be ready for use for immobilization.

Preparation of PA6/Amino acid NFs via electrospinning method

15 % (w/v) of polyamide 6 was dissolved in formic acid via magnetic stirring in ambient conditions and directly electrospun into nanofibrous mat structure with a proper electrospinning procedure. 20 % (w/v) of Glycine, Glutamic acid, and Tyrosine amino acids were added to polyamide 6 nanofibrils. Schematic illustration of the electrospinning process is demonstrated in Fig. 1 (Demirkan *et al.* 2018).

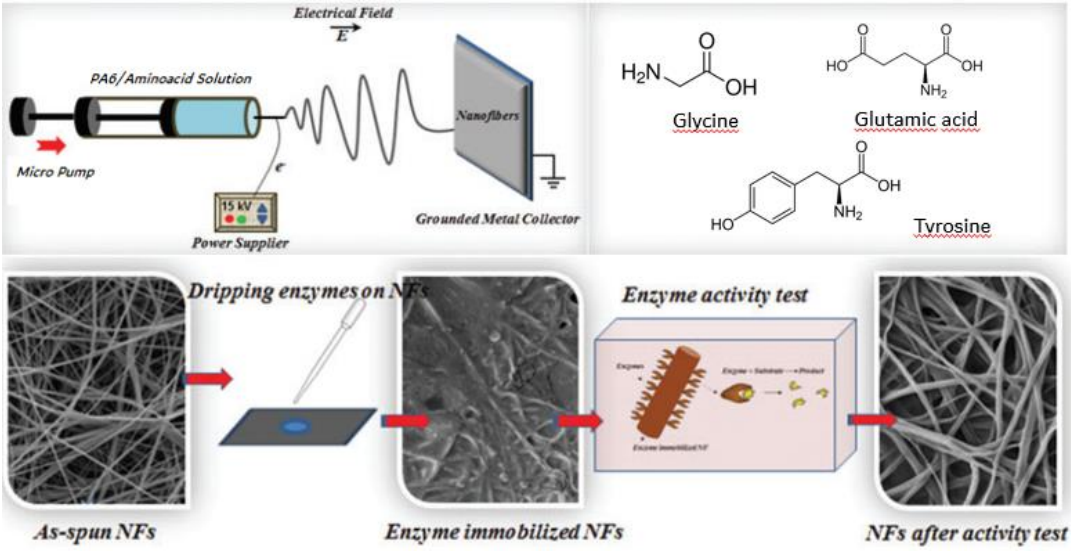

Fig. 1. Schematic illustration of the electrospinning process, chemical structures of amino acids, and the general concept of protease immobilization on as-spun nanofibrils and enzyme activity test (Demirkan *et al.* 2018).

Final stock solution (1 mL) was loaded in a plastic syringe fitted with a stainless steel needle (0.508 mm inner diameter). A metal collector was grounded and stayed in front of the needle where the polymer solution is fed. Flow rate, applied voltage and the distance were approximately 0.5 mL/h, 15 kV, and 15 cm, respectively. In order to obtain uniform NFs, these parameters were varied during the electrospinning process. Because of the applied voltage to the needle, polymer jet was ejected from the needle to grounded collector, the solvent was evaporated during the process, and dried NFs were collected on the grounded metal collector.

Characterization of PA6/Amino acid NFs

The morphologies of PA6/Amino acid NFs were analyzed using a ZEISS EVO 40 scanning electron microscope (SEM). In order to reduce charging during SEM analysis, samples were first placed on a SEM sample holder and coated with gold-palladium (about 100A˚ of thickness) using a BAL-TEC SCD005 sputter coater. Then the samples were set in the SEM chamber and SEM analyses were conducted. Acceleration voltage (20 kV) was used for SEM analysis. The viscosity analyzes of the electrospinning solutions were performed with the Anton PaaR / MCR 302 instrument.

Immobilization of enzymes on NFs

The enzymes used in the experiments were immobilized on the nanofibril surface in two different ways. In the first immobilization process, 1 mL aliquots of enzyme solutions were directly dripped onto the as-spun NF samples kept at room temperature for 12h to immobilize enzymes on the NFs. Then, enzyme immobilized NFs were vigorously washed with pure water to remove excess free protease. In the second process, proteases were immobilized on GA-activated NFs for comparison in order to observe protease immobilization on the NFs via physical adsorption. For this purpose, GA was diluted with pure water, dropped on NFs and waited for 5 min before enzyme immobilization.

The immobilization efficiency was defined as;

$$Immobilization\ efficiency\ (\%) = \left(\frac{a_{imm}}{a_{free}}\right) \times 100$$

where a_{imm} is the activity of the immobilized enzyme (U/mL) and a_{free} is the activity of the free enzyme (U/mL).

Re-usability of the immobilized enzymes

To determine the re-usability of the protease enzymes on the NFs surface, enzyme immobilized NFs were subjected to repeated hydrolysis reaction in activity assays for multiple times. Each run lasted for 20 min, after which the polymers were separated, washed with pure water and stored at 4ºC until the next use. The reaction medium was then replaced with fresh medium. Enzyme activity assays were sequential throughout the cycle. In order to prevent conditions affecting the enzyme, NFs were carefully removed when the first cycle was over and the second cycle was initiated and continued for other cycles. The activity in the first run was defined as 100 %.

Statistical analysis

All the experiments were performed in triplicate and values were expressed in terms of mean ± SD (Standart Deviation).

Results

SEM analysis of as-spun amino acid blended PA6 NFs

Morphologies of as-spun amino acid blended PA6 NFs were evaluated via low and high magnification SEM imaging (Fig. 2). In order to attaine uniform NFs, electrospinning conditions (applied voltage, needle-to-collector distance, and solution flow rates) were varied during the electrospinning process.

The NFs produced by using only pure PA6 showed a uniform and oval structure (Fig. 2. A1, A2). In these pure PA6 NFs, the diameter of the nanofibril was between 50 and 150 nm (Fig. 2. A3). Glycine doped PA6 (Fig. 2. B1, B2) NHs showed a nanofibril diameter in a wide range from 150 nm to 800 nm (Fig. 2. B3). However, the diameters of 20 % of the NFs were in the 400-450 nm range. No uniform structure was observed in NFs formed with the addition of Glycine and also bead formation occurred on NFs. PA6 (Fig. 2. C1, C2) NFs formed with Tyrosine addition were observed as uniform and oval. Nanofibril diameters were between 100-200 nm (Fig. 2. C3). Glutamic acid doped PA6 NFs showed much less bead formation than Glycine doped NFs and the resulting NFs were uniform (Fig. 2. D1, D2) and were mainly 100-200 nm in diameter (Fig. 2. D3).

Enzyme immobilization yield and stability measurement

Proteases were immobilized on as-spun NFs via the physical adsorption method and held for 12 h, and then enzyme activity measurements were performed. The immobilization efficiencies of the enzymes were approximately between 50-55 %. Immobilized enzymes were washed with pure water and stored at 4ºC until the next repetition. The initial activities were defined as 100 %. The immobilization efficiency of protease on as-spun PA6 and PA6/Amino acid NFs without treatment and with GA treatment was inspected by observing the number of reuse cycles of immobilized protease (Fig. 3). In studies with lyophilized *Bacillus* protease, glutaraldehyde activated PA6 NFs and glutaraldehyde unactivated PA6 NFs were found to be more immobilized in the presence of Glutamic acid. Although the lyophilized protease enzyme immobilized on non-glutaraldehyde activated and activated surfaces have been used 4 times, the best functional stability has been achieved with 2 times use. In pure PA6/Glutamic acid NFs, the immobilization yield of the enzymes used 2 times was found to be 38 % (Fig. 3 A1). In glutaraldehyde-activated PA6 NFs, the PA6/Glutamic acid nanofibril surfaces were found to have 65 % immobilization yield of the twice used enzymes (Fig. 3 A2).

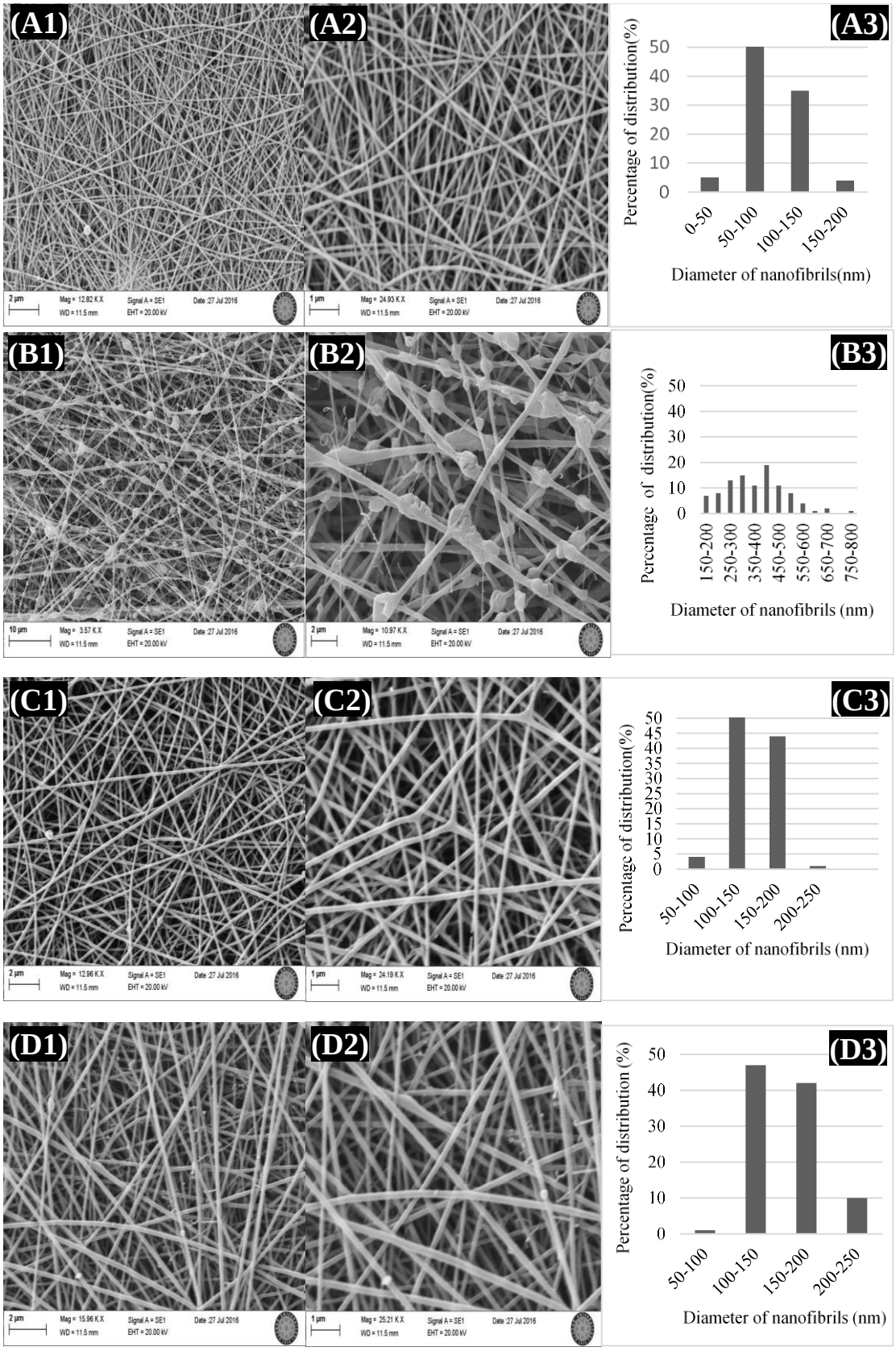

Fig. 2. SEM images of amino acid doped PA6*NF*s (A1, B1, C1, D1 with 15,96 KX magnification, A2, B2, C2, D2 with 25,22 KX magnification): (A) Pure PA6, (B) PA6 / Glycine, (C) PA6 / Tyrosine, (D) PA6 / Glutamic acid. A3, B3, C3 and D3 show diameter values of NFs.

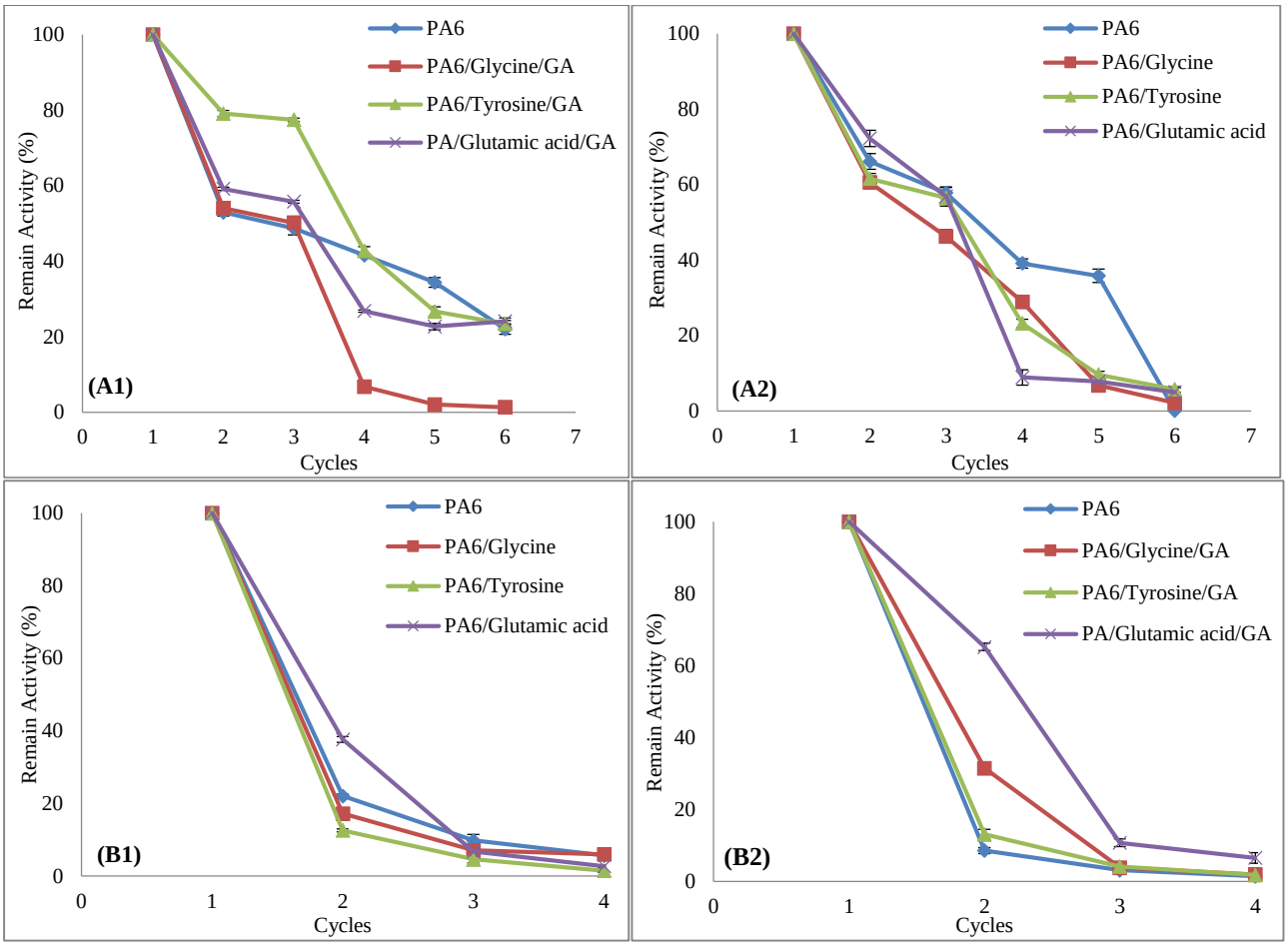

Fig. 3. Reusability of immobilized lyophilized and commercial protease enzyme. (A1) PA6/Amino acid NFs with lyophilized protease, (A2) PA6/Amino acid NFs coated with GA with lyophilized protease, (B1) PA6/Amino acid NFs with commercial protease (B2) PA6/Amino acid NFs with GA with commercial protease. All values are averages of results from triplicate trials; error bars indicate the SD values. PA6: polyamide 6; GA: glutaraldehyde; NF: nanofibril.

The enzyme immobilization efficiency has been doubled by glutaraldehyde activation of the NFs. In studies with commercial proteases, the most functional stability was obtained for 3 repeated uses, although the enzyme was used 6 times on the non-glutaraldehyde activated nanofibril surfaces. The most successful immobilization was found in 58 % of PA6 NFs (Fig. 3 B1). In glutaraldehyde-activated PA6 NFs, the enzyme was found to be used 6 times, but the functional stability was maintained as much as 4 times of repeated use. The enzyme immobilization yield in PA6/Tyrosine NFs was 43 % (Fig. 3 B2).

Discussion

Nanofibers stand out among the materials currently used in immobilization because it is widely accepted that nanoscale materials have large surface / volume ratios for enzyme loading. Electrospun NFs are promising supports for enzyme immobilization, as they have high porosity and low diffusion resistance that allow access to high specific surface areas and enzyme activity. These systems also provide easy reusability and potential applicability for continuous operations. Since PA6 is an easily available material, it is a suitable material for immobilization studies (Chen *et al.* 2004). PA6 NFs have free N-H and C=O groups. A bond may be formed between these groups and the carboxy and amino groups of amino acids (Monsan *et al.* 1975). In this study, PA6 NFs were prepared via an electrospinning process with the participation of Glycine, Tyrosine and Glutamic acid amino acids. When SEM images are examined, as in the case of pure NFs obtained in oval and uniform, Tyrosine and Glutamic acid doped NAs are also oval and uniform, but uniform structure was not observed in Glycine NFs and bead formation occurred. With the addition of amino acid, there was no obvious negative effect on the NF structure, but only too much bead formation on the Glycine NF, and less bead formation on Glutamic acid NF occurred.

Lyophilized *B. subtilis* E6-5 protease and commercial protease were immobilized on these NFs. As a result of immobilization of lyophilized enzymes on pure PA6/amino acid NFs, after 2 cycle of usage, enzyme activity was maintained at 38 % in the presence of PA6/Glutamic acid complex. In pure PA6 NF, PA6/Glycine and PA6/Tyrosine complexes, dramatic enzyme losses were detected after 2 cycle. In studies with commercial enzymes, enzyme immobilization studies

with pure NFs were compared with studies with amino acid NFs, and it was found that enzyme activity (up to 60 %) was retained in the third cycle in all three NFs with amino acids. Enzyme losses were observed in following cycles. Results in NF with Glutamic acid were better. This may be due to the benzene ring in the Tyrosine and the lack of an adequate attachment point in Glycine. Since Glutamic acid has 2 carboxyl groups, it may have made more connections with the enzyme.

The bifunctional agents can enhance the efficiency of favourable materials, such as glutaraldehyde, cyanogen bromide, and carbodiimide which are essential to stimulate the covalent binding between enzyme molecules and NFs (Chae 1998). Among these agents, glutaraldehyde is one of the most preferred agent commonly used as intermolecular crosslinking in proteins or to modify adsorbed proteins on aminated supports (Hwang 2004). The certain structure of glutaraldehyde on the support is yet under discussion, however, given the high stability of amino-glutaraldehyde bond, the formation of some kind of cycle seems to be a likely possibility (Migneault 2004). Since it is used as glutaraldehyde activating (cross-linking) agent in immobilization studies, it was also used in this study. As a result of the application of lyophilized *Bacillus* enzyme to pure PA6 and PA6/aminoacid NFs that activated with glutaraldehyde (GA): very dramatic enzyme losses were detected in 2 cycles in the NFs with PA6/GA, PA6/Tyrosine/GA and PA6/Glycine/GA. The most successful enzyme immobilization efficiency was obtained with PA6/Glutamic acid/GA NFs. Glutaraldehyde-activated PA6/Glutamic acid complex retained 65 % of its activity after 2 repeated use. The PA6/Glutamic acid complex, which was not activated by glutaraldehyde, maintained 38 % of its activity after 2 repeated use. This showed that glutaraldehyde is important for this NF. In studies conducted with commercial enzymes, it was found that while more enzyme immobilization was provided in trials without glutaraldehyde, the reuse number was 3. When glutaraldehyde was used, less enzyme immobilization occurred, but the number of re-use was 4. In pure NF with gluteraldehyde, 50 % enzyme immobilization was detected in 2 cycles. On the other hand, it has been determined that enzyme activity in nanofibers with amino acid and glutaraldehyde remained over 50 % for 3 cycles. Especially in pure NF with gluteraldehyde and NF with Tyrosine/glutaraldehyde, it was determined that enzyme activity was preserved by 43 %. It was revealed that the addition of glutaraldehyde has an important role in NFs containing amino acids.

Glutaraldehyde may react with proteins by several means such as aldol condensation or Michael-type addition (Migneault *et al.* 2004). As a result of these discrepancies and the unique characteristics of *Bacillus subtilis* protease and the commercial protease, crosslinking procedures using glutaraldehyde are largely developed through empirical observation. The choice of the enzyme-glutaraldehyde ratio, as well as their final concentration, is critical because insolubilization of the enzyme must result in minimal distortion of its structure in order to retain catalytic activity. Glutaraldehyde can react with several functional groups of proteins, such as amine, thiol, phenol, and imidazole because the most reactive amino acid side-chains are nucleophiles (Habeeb and Hiramoto 1968). The concentrations of enzyme and glutaraldehyde must be carefully considered to obtain water-insoluble enzyme derivatives via crosslinking (Zaborsky 1973).

In their studies on the immobilization of the protease enzyme obtained from *Conidiobolus macrosporus* on polyamide, Tanksale *et al.* (2001) immobilized the enzyme to nanofibrils using 1.76 % glutaraldehyde and reported yield was 58 %. If there is no overlap between the functional group of the glutaraldehyde molecule -CHO and the amino group of the amino acids used, the functional group (-CHO) of glutaraldehyde can be randomly bound to the amine groups or amino acid residues of the enzyme, which may cause a change in the enzyme configuration. In this case, the enzyme may decrease the function or the enzyme may be completely inactivated. In addition, the free amino group of the various amino acids that used, in the immobilization process can bind glutaraldehyde with the aldehyde group and prevent the enzyme from fully binding to the NF surfaces (Migneault *et al.* 2004). Study of immobilization of commercial protease enzyme on glutaraldehyde-free NFs showed that the highest activity was in the PA6/Glycine NFs. In terms of enzyme activity, PA6/Glycine was followed by pure PA6, PA6/Tyrosine and PA6/Glutamic acid NFs. Despite the high immobilization success of PA6/Glycine NFs in the first usage, pure PA6 NFs was found to be more efficient in preserving the activity. The success of immobilization of pure PA6 NFs was demonstrated by preserving 58 % of their activities up to 3 repetitive uses. In the study conducted with commercial protease enzyme, the activity loss experienced by the enzyme was investigated up to 6 repetitions. In the study performed with PA6/amino acid NFs activated with glutaraldehyde, PA6/GA complex showed the highest activity considering the initial activities. The PA6/GA complex was followed by PA6/Glutamic acid/GA, PA6/ Glycine/GA and PA6/Tyrosine/GA complexes, respectively. Enzymes immobilized PA/Tyrosine/GA NFs were considered to be the most successful immobilization, with maintaining 43 % of their activity up to 4 repetitions. Aykut *et al.* (2017) prepared cellulose monoacetate/polycaprolactone and cellulose monoacetate/ polycaprolactamand activated the surfaces with aldehyde groups for protease immobilization. They obtained 35.5 % efficiency after eight reuses, and enzyme activities of about 23 % were still observed even after nine reuses (both for GA-activated and inactivated, pure CMA NF samples). Immobilized enzymes are preferred over their native counterparts because of their potential for repetitive use. In addition, the reaction product is not contaminated with the enzyme and the immobilized enzyme has a longer half-life and predictable decay rate (Reshmi *et al.* 2006). Immobilization condition obtained in this study may find use in different fields of textile and food.

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Research Article

PARTIAL PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR METALLOPEPTIDASE PRODUCED BY *Bacillus amyloliquefaciens* FE-K1

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Metallopeptidase
Ropey bread
Purification

Abstract: The aim of this study was to purify and characterize the peptidase of *Bacillus amyloliquefaciens* (Fukumoto) (strain FE-K1) isolated from ropey bread. Peptidases were purified from crude enzyme solution by affinity chromatography with an efficiency of 25 % and a purification coefficient of 1.53. The optimum pH of partially purified peptidase (PPPase) solution was determined as 7.5 and the peptidases retained approximately 90 % of their initial activity in the pH range 7.0-8.5 following incubation at 37°C for 2 h. The optimum temperature for the PPPase was 60°C. The approximate molecular weight of the PPPase was determined as 36 kDa. Inactivation of the PPPase in the presence of O-FEN and EDTA showed them to be metallopeptidases and 5 mM of K^{+1} and 5 mM of Mn^{+2} ions increased the enzyme activity by 4 % and 6.15 %, respectively. The presence of Hg^{+2}, Fe^{+3} and SDS (0.1-1.0 % w/v) caused inactivation whereas the enzyme retained most of its activity in the presence of 0.1-1.0 % (v/v) Triton X-100, Tween 20 and Tween 80 and 1-20 % (v/v) xylene, ethanol, acetone and acetonitrile. Characterization of the PPPase revealed the enzyme as a neutral serine metallopeptidase compatible with some organic solvents and surfactants.

Özet: Bu çalışmanın amacı, sünmüş ekmeklerden izole edilen *Bacillus amyloliquefaciens* (Fukumoto) (suş FE-K1) ile elde edilen peptidazı saflaştırmak ve karakterize etmektir. Peptidazlar ham enzim çözeltisinden afinite kromatografisi ile % 25 verim ve 1,53 saflaştırma katsayısı ile saflaştırılmıştır. Kısmi olarak saflaştırılmış peptidaz (PPPaz) çözeltisinin optimum pH değeri 7,5 olarak tespit edilmiş olup, pH 7,5-8,0 aralığında peptidaz, 37°C'de 2 saat inkübasyonun ardından başlangıç aktivitesini yaklaşık % 90 oranında korumuştur. PPPaz'ın optimum sıcaklığı 60°C'dir. PPPaz'ın yaklaşık molekül ağırlığı 36 kDa olarak belirlenmiştir. PPPaz'ın O-FEN ve EDTA varlığında inaktive olması, enzimin metallopeptidaz olduğunu göstermiştir. Ayrıca 5 mM K^{+1} ve 5 mM Mn^{+2}, enzimin aktivitesini sırasıyla % 4 ve % 6,15 oranında artırmıştır. Hg^{+2}, Fe^{+3} ve SDS (% 0,1-1,0 w/v) varlığı enzimin inaktivasyonuna neden olurken, % 0,1-1,0 (v/v) Triton X-100, Tween 20 ve Tween 80; ve % 1-20 (v/v) ksilen, etanol, aseton ve asetonitril varlığında enzim aktivitesini büyük ölçüde korumuştur. PPPaz'ın karakterizasyonu, enzimin bazı organik çözücü ve yüzey aktif maddelerle ile uyumlu nötr bir serin metalopeptidaz olduğunu ortaya çıkarmıştır.

Introduction

Peptidases (EC 3.4) constitute an enzyme group that converts proteins or large polypeptides into smaller peptides or free amino acids by hydrolysing peptide bonds (da Silva 2017). Although generally known as proteases, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends using the terms 'peptidase' or 'peptide hydrolase' for any enzyme that hydrolyses peptide bonds (Rawlings *et al*. 2007).

Peptidases are essential constituents of all life forms on earth, including prokaryotes, fungi, plants and animals, and therefore can be used for industrial purposes when isolated from these organisms (Gupta *et al*. 2002a, Gupta *et al*. 2002b). These enzymes sourced almost exclusively from microorganisms have extensive applications in food industry, pharmaceutical industry, detergent industry, bioremediation processes, and leather treatment, among others (Sandhya *et al*. 2005, Razzaq *et al*. 2019, Salwan & Sharma 2019, Thapa *et al*. 2019). Therefore, many

researchers pay much of their attentions to these enzymes and are focusing on discovering and developing new peptidases with desired properties.

Microorganisms secrete peptidases to degrade proteins and utilize the degradation products as nutrients for their growth. Degradation is initiated by endopeptidases and is followed by exopeptidases at extra- or intracellular locations for further hydrolysis. A variety of peptidases are produced by microorganisms depending on the species of the producer or the strain. The type and quantity of enzyme produced may vary for different strains of the same species or even the same strain under different cultural conditions (Sandhya *et al*. 2005, Jisha *et al*. 2013). Despite the long list of peptidase-producing microorganisms, only a few being considered as potential producers for commercial exploitation are 'generally recognised as safe' (GRAS), non-toxic and non-pathogenic. Of these, strains of *Bacillus* spp. dominate the industry producing a substantial portion of all extracellular peptidases used. Furthermore, these strains are quick to grow and much is known about their biochemistry, physiology and genetics [especially *Bacillus subtilis* (Ehrenberg) Cohn], which facilitates further development and greater exploitation of these microorganisms in industrial processes (Waites *et al*. 2001, Gupta *et al*. 2002a, Schallmey *et al*. 2004, Contesini *et al*. 2018).

Rope is a type of spoilage seen in bread caused by *B. subtilis* as the most common causative agent along with *B. licheniformis* Weigmann, *B. megaterium* Bary, *B. pumilus* Meyer and Gottheil, and *B. cereus* Frankland and Frankland. During the baking process, most vegetative forms of bacteria are killed. However, spores can endure the baking process, where the temperature in the centre of the crumb may not rise above 100°C. Under humid and warm storage conditions the spores germinate and cause rope spoilage (Kirschner & von Holy 1989, Collins *et al*. 1991, Volavsek *et al*. 1992, Thompson *et al*. 1993). The most apparent change takes place in the centre of the crumb, observed as stickiness and development of putrid odor. Stickness develops as a result of degradation of starch and protein molecules via amylases and peptidases secreted by the germinated bacteria (Kirschner & von Holy 1989, Voysey 1989, Bailey & von Holy 1993, Rosenkvist & Hansen 1995, Ellis *et al*. 1997, Thompson *et al*. 1998, Pepe *et al*. 2003, Erem *et al*. 2009). Therefore, *Bacillus* spp. isolated from ropey bread are candidate strains, which may be exploited for industrial peptidase production. The aim of this study was to purify and characterize the peptidase of *B. amyloliquefaciens* FE-K1 previously isolated from ropey wholemeal bread and to evaluate the suitability of the peptidase produced for industrial use based on some of its characteristics.

Materials and Methods

Microorganism and the growth media

The bacterial strain used in this study was previously isolated from ropey wholemeal bread (Erem *et al*. 2009). The gene sequence of the strain was determined with 16S rRNA by REFGEN (Gene Research and Biotechnology, Ankara, TURKEY), and the strain was identified as *B. amyloliquefaciens* FE-K1. Nucleotide sequence data for the strain are available in the GenBank databases under the accession number MH045777. Furthermore, *B. amyloliquefaciens* FE-K1 was verified as a haemolytic (Hbl) and non-haemolytic (Nhe) enterotoxin-negative strain (not published).

The composition of the growth medium (GM1) used for the preparation of preculture was 2 g/L of glucose, 10 g/L of yeast extract, 1 g/L of KH_2PO_4, 3 g/L of $K_2HPO_4.3H_2O$, 2 g/L of Na_2SO_4 and 0.1 g/L of $MgSO_4.7H_2O$. The pH of GM1 was 7.2, but; it was adjusted to 6.62 (Erem *et al*. 2018) with 6N HCl and 6N NaOH before sterilization and used as the primary peptidase production medium (GM2). Stock culture and inoculum were prepared according to Erem *et al*. (2018).

Peptidase production

The cultivation for enzyme production was done with 2.3 % (v/v) of preculture in flasks containing GM2 at 33.4°C and 250 rpm for 7 h. The inoculation rate, temperature, shaking rate, and the time previously determined as optimum levels for enzyme production were used as cultivation parameters (Erem *et al*. 2018, Erem & Certel 2018). Cell-free supernatant obtained by centrifugation (at 20000 × *g*, 4°C for 15 min) was used as the crude enzyme solution (CES).

Peptidase assay

The peptidase assay was performed according to Cupp-Enyard (2008) with some modifications. After incubation, a clear solution was obtained by centrifugation at 10000 × *g* and 4°C instead of using syringe filters. The peptidase activity of cell-free clear supernatant was assayed at 37°C in potassium phosphate buffer (50 mM, pH 7.5) by using 0.65 % (w/v) casein as substrate. One unit of peptidase was equivalent to the amount of enzyme required to release 1 mg of tyrosine/mL/min under standard assay conditions.

Protein content

The total protein content of the enzyme solution was determined by the method of Bradford (1976) using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific, Pierce Biotechnology, USA).

Pretreatment of solutions and CES before purification

All the solutions used in the purification process were prepared with double-distilled water. They were filtered through a 0.22 µm filter (PES) and degassed in an ultrasonic water bath (Bandelin Sonorex RK 255 H, Germany). CES was filtered through a 0.45 µm filter (PES, low protein-binding). Then, 15 mL of filtered CES was put into an ultrafiltration tube (Vivaspin 20, 10000 MWCO, PES, Sartorious) and centrifuged (at 8000 × *g*, 4°C) until 10-fold volumetric concentration was achieved. Concentrated CES (1.5 mL) was loaded onto a HiTrap desalting column (GE Healthcare, Sweden) for buffer exchange using an Äkta Prime purification system (GE

Healthcare Life Sciences, Sweden) with a flow rate of 5 mL/min. Column equilibration and the buffer exchange process were made with 100 mM 3-(N-morpholino) propanesulfonic acid [MOPS (pH 7.0)]. The effluent from the column was monitored with a UV monitor during the buffer exchange process, and 1 mL of fractions was collected until the UV peak gave an absorbance of zero. Peptidase analysis was performed on each fraction, and the fractions showing peptidase activity were combined for the main purification.

Purification of the peptidase by affinity chromatography

Column and buffer solutions were selected according to Lauer *et al*. (2000). Purification was made with the Äkta Prime protein purification system by using 1 mL of a HiTrap Blue HP column (GE Healthcare, Sweden). After the column was equilibrated with the binding buffer of 100 mM MOPS (pH 7.0), 5 mL of the sample was loaded onto the column with a flow rate of 1 mL/min. Then, the column was washed with 10 mL of the binding buffer. Later, the column was washed with 25 mL of elution buffer (100 mM MOPS, 1 M NaCl, pH 7.0) by using a step gradient. One mL of fractions collected from the beginning of sample loading until the end of the elution process were tested for peptidase activity. However, peptidase activity analysis of elution fractions was performed after the desalting procedure using a HiTrap desalting column with a buffer solution of potassium phosphate (50 mM, pH 7.5). Fractions that showed peptidase activity were used for further analysis.

Electrophoresis and zymography

SDS-PAGE was carried out by the method described by Laemmli (1970) using 10 % polyacrylamide resolving gels. Staining was done by Coomassie Blue (G-250). Molecular weight markers of 10-200 kDa (Page Ruler Unstained Protein Ladder, Pierce Biotechnology, USA) were used. The gels were scanned with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

Zymography was performed by modifying the method of Leber & Balkwill (1997). Peptidases were separated on SDS-PAGE gels containing 0.08 % casein (w/v) as substrate. After electrophoresis, gels were treated as described by Fernández-Resa *et al*. (1994). Staining and visualization were the same as for SDS-PAGE gels. SDS-PAGE and zymography were also applied to desalted samples of elution fractions.

Determination of optimum pH value of the enzyme and pH stability

The peptidase activity was analysed at 37°C with a substrate of 0.65 % (w/v) casein prepared in 50 mM of different buffer systems [sodium citrate (pH 6), potassium phosphate (pH 7.0 and 7.5), Tris-HCl (pH 8.0, 8.5 and 9.0), glycine-NaOH (pH 10 and 11) and KCl-NaOH (pH 12 and 13)]. After determining the optimum pH value, all other characterization tests were carried out at this pH value.

Peptidase solution was mixed with an equal volume of the buffer systems mentioned above and incubated at 37°C for the determination of pH stability. The peptidase activity of these mixtures was measured immediately before and after the incubation (2 and 18 h). The peptidase activity of the mixtures measured before the incubation was accepted as 100 %, and the activity after the incubation was calculated as the activity remaining (%). The purpose of selecting 37°C as the incubation temperature before the activity test is the stability of the enzyme at this temperature.

Determination of optimum temperature of enzyme and temperature stability

The optimum temperature of peptidase was determined by measuring the enzyme activity at a temperature range of 4-70°C with a substrate of 0.65 % (w/v) casein prepared in phosphate buffer (50 mM, pH 7.5). After determining the optimum temperature, all other characterizations were carried out at this temperature.

In order to determine the temperature stability of peptidase, peptidase solutions were preincubated at different temperatures between 4 and 70°C, and during incubation, samples were taken at various time intervals (10, 30, 60, 90 and 120 min) to test for peptidase activity. The peptidase activity of non-incubated enzyme solutions was accepted as 100 %, and the remaining activity of the incubated enzyme solutions was calculated as a percentage.

Effect of peptidase inhibitors

Enzyme solutions were mixed with different concentrations of peptidase inhibitors [final concentrations after mixing: (PEP-A: 5, 10, 20 µM), (E-64: 10, 20, 40 µM), (PMSF, O-FEN, and EDTA: 1, 2, 4 mM)] and incubated at 37°C for 30 min. Then, peptidase activity was measured (at 60°C, pH 7.5), and calculations were made by accepting the activity of inhibitor-free enzyme solutions as 100 %.

Effect of metal ions

Enzyme solutions were mixed with equal volumes of metal ions ($BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $MgCl_2$, $HgCl_2$, $MnCl_2$, $ZnCl_2$, $NiCl_2$, KCl and NaCl) to a final concentration of 0.5, 2.0 and 5 mM. Then, they were incubated at 37°C for 30 min. Peptidase activity was measured (at 60°C, pH 7.5), and the activity of enzyme solutions diluted with distilled water (1:1) and incubated at the same conditions was accepted as 100 %. The relative activity of metal ion-containing enzyme solutions was calculated.

Effect of organic solvents and surfactants

In order to determine the effects of organic solvents [ethanol, acetone, acetonitrile and xylene: 1, 5, 20 % (v/v)] and surfactants [Triton X-100 (v/v) and SDS (w/v): 0.1, 0.5, 1.0 %; Tween 20 and Tween 80: 0.1, 0.5, 1.0 % (v/v)] on enzyme activity, enzyme solutions were mixed with these substances to the final concentrations given and

then incubated at 37°C for 30 min. After incubation, the peptidase activity was tested at optimum temperature (60°C) and pH (7.5). The activity of substance-free enzyme solutions was accepted as 100 %, and the activity of others was calculated relatively.

Statistical evaluation

Experiments were carried out with duplicate samples and triplicate analysis, and the data given represent mean values ± standard deviations. Correlation between inhibitor concentration and peptidase activity was determined with Minitab Statistical Software (Version 18, Minitab Inc. USA).

Results and Discussion

Production of peptidase by B. amyloliquefaciens FE-K1

The optimum culture conditions of *B. amyloliquefaciens* FE-K1 for producing peptidase have previously been determined by using one factor at a time (Erem & Certel 2018), and response surface methodologies (Erem *et al*. 2018). Depending on the results of our previous studies, *B. amyloliquefaciens* FE-K1 peptidase was produced by inoculating 2.3 % (v/v) of preculture into flasks containing the production medium and growth at 33.4°C and 250 rpm for 7 h. At the end of the cultivation, the peptidase activity and the protein content of the CES were determined as 40.74 ± 5.48 U/mL and 0.12 ± 0.04 mg/ml, respectively, for three different production batch, which gave the best results among the other batches. After ultrafiltration, the peptidase activity and the protein content of the concentrated CES were found as 233.91 ± 25.85 U/mL and 0.57 ± 0.49 mg/ml, respectively. Each batch was evaluated separately in the chromatographic purification steps.

Purification of peptidase by affinity chromatography

Bacillus amyloliquefaciens FE-K1 peptidase was purified by two chromatographic steps. Before its application to the columns, the CES (35.12 ± 0.16 U/mL) was concentrated, and the activity increased to 201.83 ± 2.16 U/mL. Then, the concentrated CES was loaded onto a HiTrap desalting column to exchange the buffer solution. A total of 15 fractions were collected during buffer exchange, and they were analysed for their peptidase activity. As seen in Figure 1a, among the total 15 fractions, peptidase activity was observed only in the first six fractions and, of these fractions, the second (140.86 ± 2.36 U/mL) and third (108.78 ± 2.95 U/mL) showed the highest activity. Therefore, it was decided to use a combination of these six fractions, which showed peptidase activity for the main purification step.

Although the primary purpose of the buffer exchange process was to adapt the enzyme solution to the binding buffer (100 mM MOPS, pH 7) used in the affinity chromatography as the initial buffer, this step also provided partial purification of the enzyme. According to Fig. 1a, only the first few fractions had peptidase activity, but UV absorbance values were still high up to the 12th fraction. This implies that there were also proteins in the enzyme solution other than peptidases which can leave the column after the peptidases due to the molecular weight difference. In this context, peptidase could be separated from some smaller peptide/protein molecules and possibly other molecules with the process of buffer exchange.

Changes of some parameters related to purification of the peptidase by the HiTrap Blue HP column are shown in Fig. 1b. It was observed from the UV plot that the absorbance was high during the passage of the sample through the column and at the beginning of the washing process. Shortly after the elution stage started, another peak (fractions 16-18) was observed in the UV plot, an indicator of protein presence. This situation showed that there were also bound proteins in the column, which were eluted with the elution buffer.

A total of 26 fractions were collected during purification, and all were analysed in terms of peptidase activity. As seen in Fig. 1b, the fractions which had high absorbance values (UV plot) also showed peptidase activity. Activity results showed that a considerable amount of the peptidase could not bind to the column and passed through the column directly. Furthermore, the peptidase molecules which had stayed in the column left it as soon as the washing stage started. Moreover, the peptidase activity of the elution fractions showed that a small amount of the enzyme could bind to the column.

SDS-PAGE results are shown in Fig. 2. All of the fractions collected were loaded onto the gels (the fractions for which a protein band could not be observed are not shown in the figure). According to Fig. 2, there are protein bands at the level of approximately 36 kDa from the beginning of the CES to the eighth fraction. While the intensity of the bands is clear for the second to sixth fractions, a decrease in band intensity was observed for the seventh and eighth fractions. Furthermore, the band intensity of the fractions was also well correlated with their peptidase activity. Therefore, it was thought that these 36 kDa bands most probably belonged to peptidase. On the other hand, the presence of protein bands other than at 36 kDa indicates partial purification.

A 36 kDa protein band could not be observed for the fractions collected during elution (16-18) which implies that this protein could not bind to the column. However, there were protein bands at the level of 100 kDa. Therefore, it can be said that the enzyme solution contained different types of peptidase. In order to make a comparison, elution fractions 16-18 were loaded onto the SDS-PAGE gels again without desalting (Fig. 2 gel b) and 36 kDa protein bands were observed with this method. The reason why bands could not be observed in the gel was probably due to the 3.6-fold dilution that occurred during desalting.

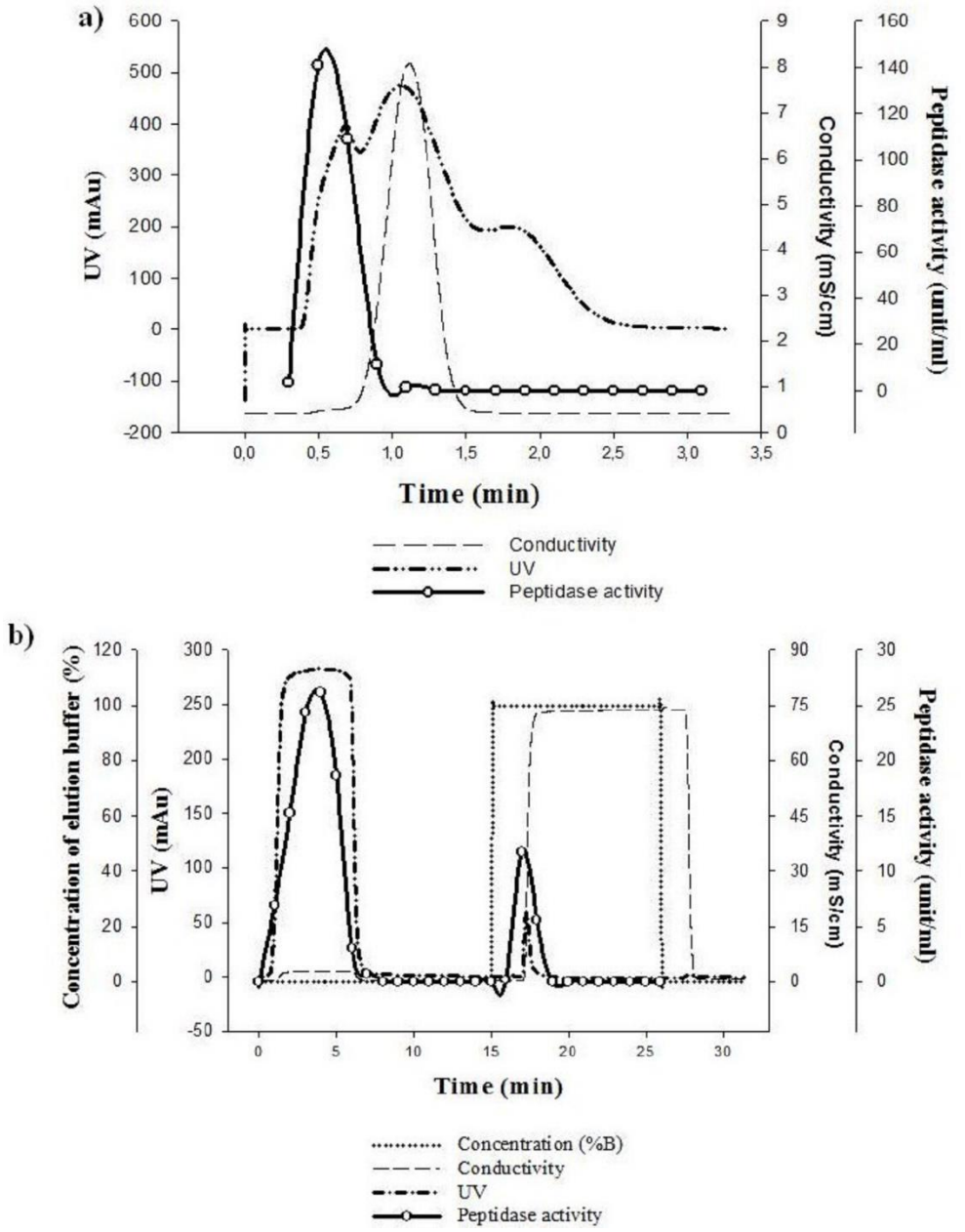

Fig. 1. Changes in conductivity, UV (absorbance), and peptidase activity values during the passage of concentrated crude enzyme solution through a) HiTrap desalting column and b) HiTrap Blue HP column.

A zymogram gel image is also shown in Fig. 2. It was observed that there were no bands at the level of both 36 kDa and 100 kDa for the elution fractions. Moreover, the right part of the gel was almost entirely dark indicating that the casein in the gel did not degrade. In other words, there was no observable hydrolysis in the gel related to the proteolytic activity for elution fractions 17 and 18, although it is known that these fractions had peptidase activity according to the spectrophotometric analysis.

The clear bands at the level of 36 kDa in Fig. 2 indicate peptidase activity, and the band intensities are also in good correlation with the activity results given as U/mL (Fig. 1b). There are also clear bands at the level of 15 kDa, and this situation points out that the CES may also contain different types of peptidase.

A remarkable subject related to the gel images (Fig. 2) was the concentrated enzyme solution, which did not show a protein band at the molecular weight level of 36 kDa in the SDS-PAGE gel but did show a clear band in the zymogram at the same level as an indicator of peptidase activity. The probable reason for this situation was thought to be the heating treatment, which was the only difference in the processes applied for SDS-PAGE and zymography samples. Owing to the high protein concentration, the conformational structure of the protein in the concentrated enzyme solution could change or be damaged.

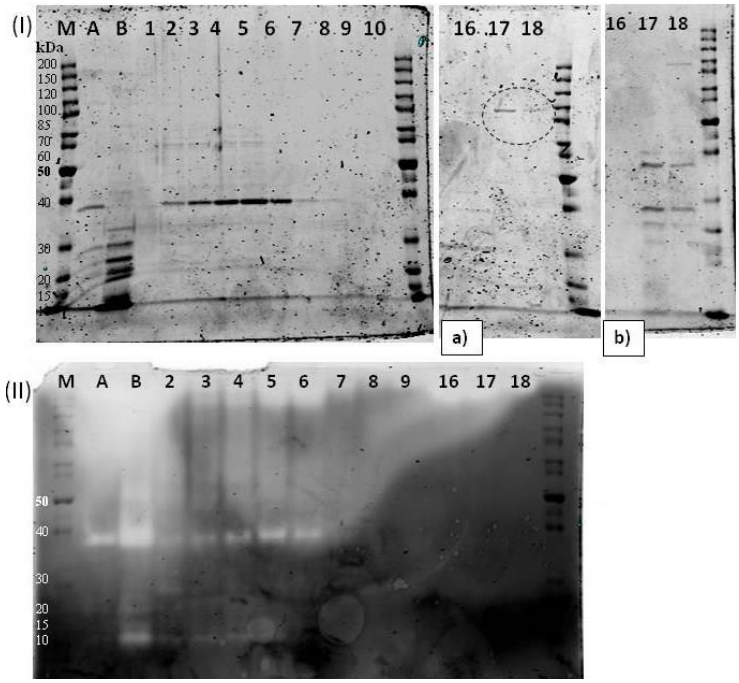

Fig. 2. (I): SDS-PAGE and (II): zymogram image of fractions collected during purification. (M: marker; A: crude enzyme solution; B: concentrated enzyme solution; 1-18: fraction numbers; a) desalting applied; b) fractions without desalting.

Table 1. Peptidase activity levels of the combined fractions after purification with a HiTrap Blue column and the activity remaining (%) after mixing peptidase inhibitors.

Fraction		Peptidase activity (U/mL)	Specific activity (unit/mg)	Inhibitor[1]			
				PEP-A (20 µM)	E-64 (40 µM)	O-FEN (4 mM)	PMSF (4 mM)
A		46.96 ± 0.66	582.56	86.40 ± 0.56	73.19 ± 0.28	13.02 ± 1.17	63.60 ± 0.08
B		248.08 ± 15.32	1717.30	84.72 ± 0.40	90.03 ± 0.32	22.88 ± 0.65	30.42 ± 0.17
C		60.79 ± 0.16	682.43	100.64 ± 2.20	92.23 ± 1.68	12.90 ± 0.00	71.43 ± 0.97
Sample loading	**1-2**	0	-	-	-	-	-
	3-4-5	23.77 ± 0.57	488.10	98.36 ± 0.41	101.17 ± 0.74	0.25 ± 0.00	89.19 ± 0.50
Washing step	**6-7-8**	25.38 ± 0.92	649.43	102.68 ± 0.00	91.35 ± 1.16	0.78 ± 0.00	91.30 ± 0.46
	9-15	1.74 ± 0.02	*	94.40 ± 0.0	96.00 ± 4.52	0.00	58.46 ± 1.13
Elution step	**16**	0	-	-	-	-	-
	17-18	19.08 ± 0.06	*	94.17 ± 0.82	88.35 ± 1.92	25.85 ± 0.27	47.59 ± 0.82

[1]The inhibitor concentrations are the final concentrations reached after mixing the enzyme and the inhibitors; activity was measured after the enzyme solution and inhibitors were mixed and kept for 30 min at room temperature. Calculations were made by accepting the activity of the samples without inhibitor as 100 %.

A: crude enzyme solution; B: concentrated enzyme solution (with ultrafiltration); C: combined fractions (1-6) during buffer exchange process (HiTrap desalting column); sample loading, washing step, and elution step are for HiTrap Blue HP column.

*Protein content could not be determined due to the very low concentration; consequently, specific activity could not be calculated.

Table 2. Peptidase purification profile.

	Volume (mL)	Peptidase activity (U/mL)	Protein (mg/mL)	Specific activity (unit/mg)	Total activity (Unit)	Efficiency (%)	Purification coefficient
A	50.00	40.13 ± 2.86	0.15 ± 0.00	265.10	2006.57	100.00	1.00
B	5.00	251.25 ± 0.80	0.99 ± 0.01	253.35	1256.23	62.61	0.96
C	21.33	49.06 ± 0.45	0.13 ± 0.02	365.90	1046.59	52.16	1.38
D	25.06	20.26 ± 0.00	0.05 ± 0.00	405.14	518.58	25.84	1.53

A: crude enzyme solution; B: concentrated enzyme solution (with ultrafiltration); C: combined fractions during buffer exchange (1-6); D: combined fractions during affinity chromatography (3-8).

Specific activity, total activity, efficiency, and purification coefficient were calculated by using mean values of peptidase activity and protein content.

Depending on the peptidase activity level of the fractions, some of the fractions were combined, and the peptidase and specific activity of these combined fractions were determined (Table 1). According to Table 1, fractions 3-8 were the best in terms of peptidase activity. However, the higher level of specific activity for fractions 6-8 indicates better purification of these fractions. Peptidase activity was also high for elution fractions 17 and 18, but their specific activity could not be calculated since the protein content was quite low.

As previously expressed, the crude enzyme solution is thought to contain different types of peptidase. In order to determine this, combined fractions were mixed with peptidase inhibitors and analysed for peptidase activity again. Activity levels were calculated relatively and are shown as remaining activity in Table 1. The substantial decrease observed in activity level in the presence of O-FEN showed that the major peptidase in all of the fractions was a metallopeptidase. In the literature, the molecular weights of metallopeptidases produced by *Bacillus* species are 19 (Sabirova *et al*. 2010), 23.5 (Choi *et al*. 2017), 30 (Matta & Punj 1998), 36 (Manni *et al.* 2008), 37.6 (See *et al*. 2018), 38 (Lauer *et al*. 2000), 40.08 (Tian *et al*. 2019), 45 (Sousa *et al.* 2007), 60 (Lee *et al*. 2016), 134 (Lee *et al*. 2002), 210 and 215 kDa (D'Costa *et al*. 2013). In this study, because the major peptidase was determined as a metallopeptidase, the clear band at the molecular level of 36 kDa in the zymogram probably belonged to a metallopeptidase. Furthermore, the observation of a decrease in activity in the presence of PMSF refers to the presence of serine peptidases in the crude enzyme solution (especially in the elution fractions).

As shown in Table 1, the peptidase activity of fractions 3-5, 6-8, and 9-15 was almost completely lost in the presence of O-FEN. Because these fractions were metallopeptidase-rich, it was decided to use a combination of them in the characterization studies except the fractions 9-15, which have very low peptidase activity (1.7 U/mL) and could have caused dilution. Therefore, only fractions 3-8 were used in the characterization studies.

Although there were elution fractions that showed peptidase activity, they were not used in the characterization studies since no band could be determined for them in the zymogram. Moreover, the specific activity could not be calculated and, consequently, it was not possible to determine the purity level. The peptidase purification profile (from crude enzyme to fractions 3-8) is shown in Table 2. It can be seen that only partial purification could be obtained, with a purification coefficient of 1.53 and efficiency of 25.84 %. Sousa *et al*. (2007) purified *B. cereus* metallopeptidase with a three-step chromatographic procedure and determined a purification coefficient and efficiency as 57.6 and 2.16 %, respectively. They obtained good purification despite the low-efficiency level. Manni *et al*. (2008) also used three-step chromatographic purification after an ultrafiltration step for the metallopeptidase of *B. cereus* SV1 and obtained a similar efficiency level (27.74 %) as in our study. However, the purification coefficient was determined as 6.09. In another study, *Bacillus subtilis* FBL-1 peptidase was purified 94.89-fold with a yield of 2.3 % (Si *et al*. 2018).

Peptidase characterization

Characterization studies were applied both for CES and the combined fractions 3-8 (partially purified peptidase; PPPase) to obtain a comparison.

Optimum pH of peptidase and pH stability

The peptidase activity results for CES and PPPase at different pH values (6.0-13.0) are shown in Fig. 3. It is obvious from the results that despite the activity loss in PPPase, the activity profiles of both CES and PPPase, exhibited at different pH values, are quite similar. The optimum peptidase activity was obtained at pH 7.5 for both CES (50.28 U/mL) and PPPase (25.57 U/mL). The activity decreased at lower and higher pH levels. Generally, the optimum activity for metallopeptidases is in the pH range between 7.0 and 8.0 (Rao *et al*. 1998, Salleh *et al*. 2006). Furthermore, Salleh *et al*. (2006) declared that metallopeptidases are the most unstable type of peptidase and can undergo autolysis quickly in the pH ranges below 6.0 and above 9.0. Si *et al*. (2018) obtained the highest activity at pH 9.0 for *B. subtilis* FBL-1 metallopeptidase and observed a sharp decrease in the enzyme activity above this value.

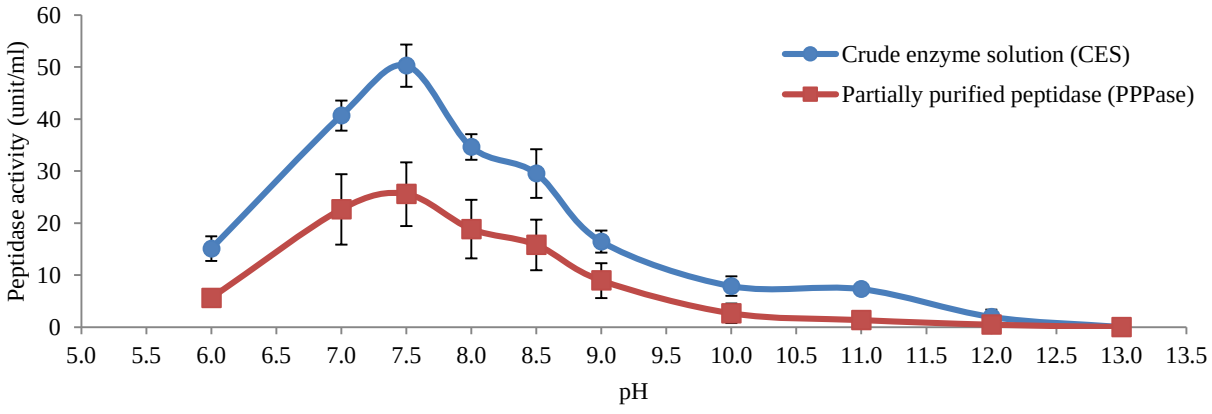

Fig. 3. Peptidase activity results at different pH levels.

On the other hand, Sharmila *et al*. (2018) determined pH 8.0 as the optimum pH level for a calcium-dependent peptidase from *B. subtilis* CFR5. The optimum pH value for *B. cereus* metallopeptidase was determined as 7.0 and 8.0 by Sousa *et al*. (2007) and Manni *et al*. (2008), respectively. Matta & Punj (1998) determined the optimum pH level for *B. polymyxa* B-17 as 7.5. The major peptidase in enzyme solutions obtained in our study was a metallopeptidase. Therefore, the results are in accordance with the literature data. Additionally, while CES retained 80.9 % and 68.9 % of its initial peptidase activity at pH 7.0 and 8.0, respectively, the closest pH levels to the optimum pH, PPPase retained 88.6 % and 73.7 % of its initial peptidase activity in the same conditions.

The stability of the produced peptidase solutions at different pH levels are given as remaining activity (%) in Fig. 4. According to the results, the peptidase retained a considerable amount of its initial activity in the pH range 7.0-9.0 after incubation at 37°C for 2 h (Fig. 4a). However, a substantial decrease in peptidase activity was observed especially at pH values higher than 9.0. In the case of a longer incubation period (18 h), CES showed stability by retaining approximately 75-80 % of its initial activity in the pH range 7.0-8.0; on the other hand, PPPase could only retain 27 % and 63 % of its activity at pH 7.0 and 8.0, respectively. Although the optimum pH level was 7.5 for PPPase, it retained its activity for a longer period of time at pH 8.0. This situation could be attributed to the possible presence of another type of peptidases, most probably serine peptidases, which might be in the enzyme solutions. As shown in Table 1, the presence of PMSF, which is the inhibitor of serine peptidases, caused a decrease in the peptidase activity. It was determined that serine peptidases are generally stable at pH 8.0 (Mothe & Sultanpuram 2016, Hammami *et al*. 2020). It was observed that the stability of the enzyme decreased in alkaline conditions, especially for more extended incubation periods (Fig. 4b). However, the stability of CES was better than that of PPPase, probably because it contains different types of peptidase, which may have higher optimum pH levels. Choi *et al*. (2017) observed that the purified serine metallopeptidase was stable in a pH range of 5.5-8.0 at 36°C for 2 h.

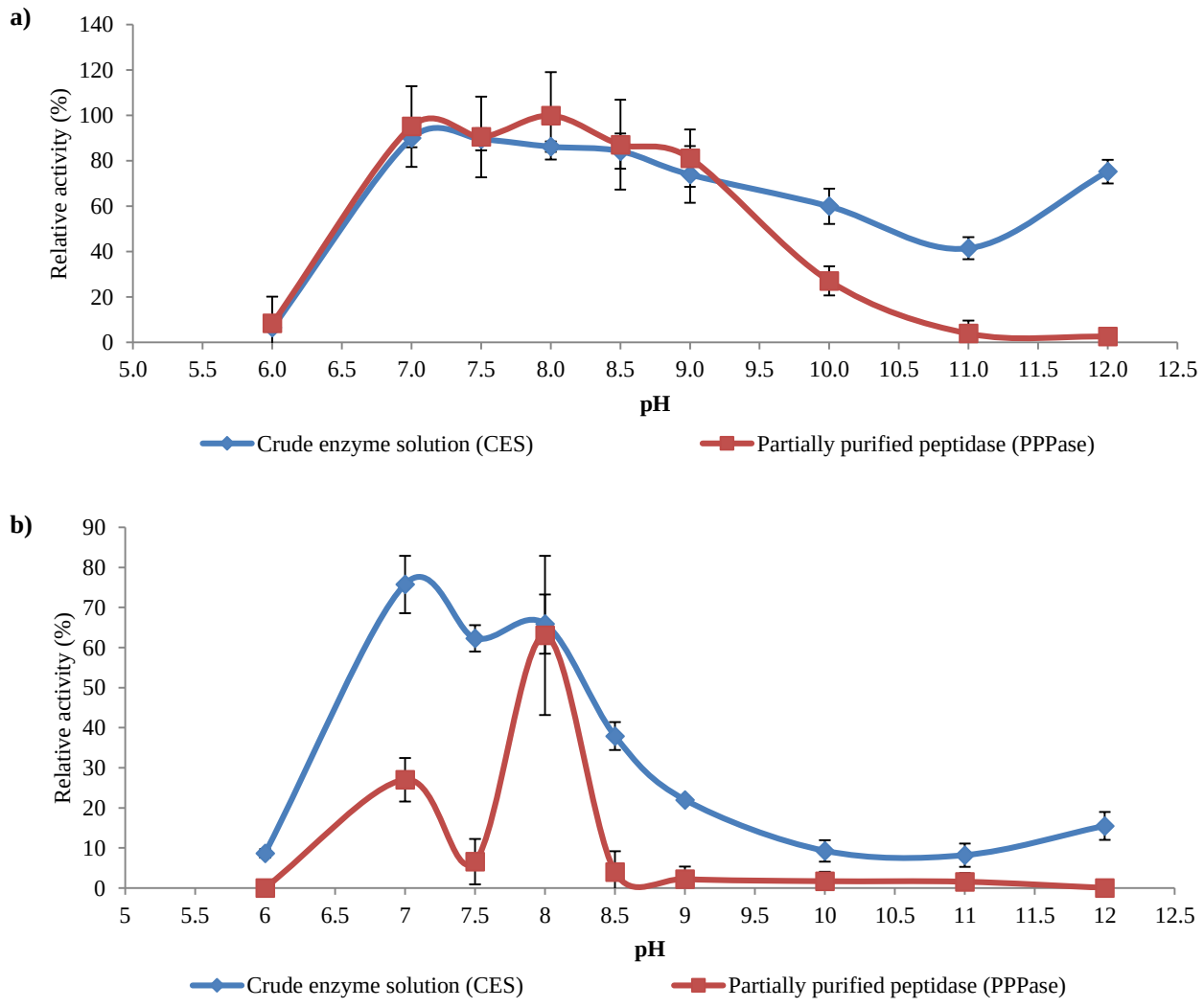

Fig. 4. pH stability of peptidase after a) 2 h and b) 18 h of incubation.

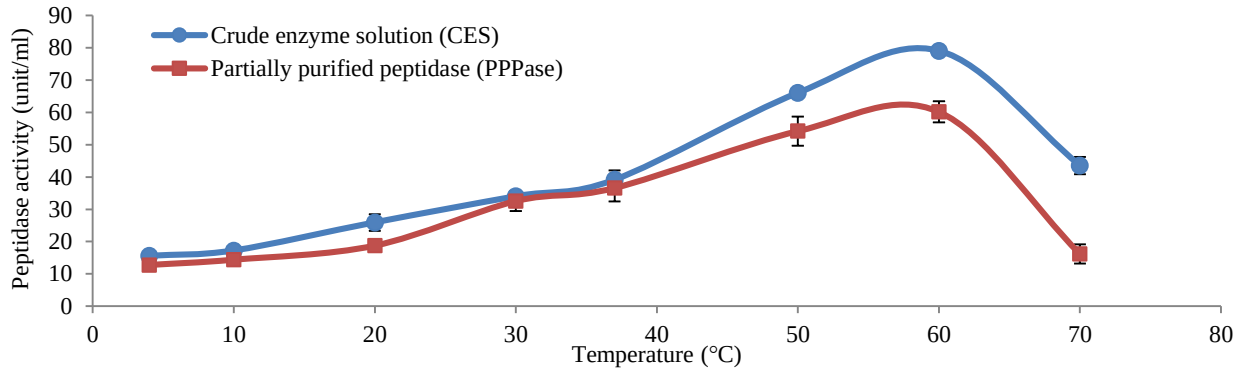

Fig. 5. Peptidase activity results at different temperatures.

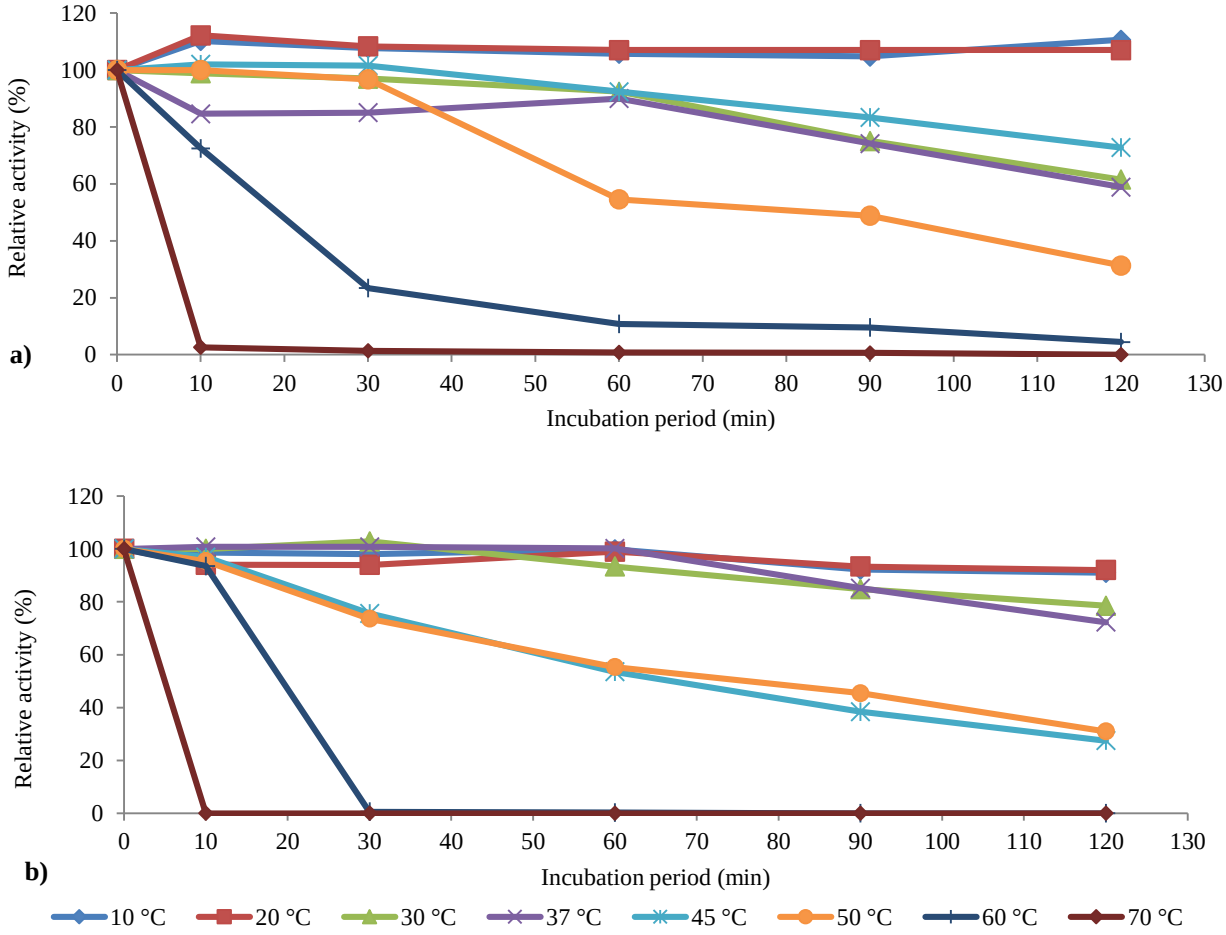

Fig. 6. Stability of a) CES and b) PPPase at different temperatures.

Manni *et al*. (2008) determined that metallopeptidase retained its initial activity almost entirely in the pH range 7.5-9.0 at 30°C for 15 min. According to a previous study (Ghorbel *et al*. 2003), a crude peptidase solution containing metallopeptidase retained more than 95 % of its initial activity after incubation at 50°C for 1 and 3 h at pH 8.0. In our study, CES showed 75-80 % stability after 18 h of incubation, which can be evaluated as having good pH stability.

A surprising result was obtained at pH 12.0. While PPPase was inactivated at this pH level, CES retained approximately 75.26 % of its activity after 2 h of incubation despite having a much lower activity level of 1.9 U/mL. This was probably due to the serine peptidase presence in CES. As declared earlier (Table 1), the CES peptidase activity remaining in the presence of PMSF was approximately 64 %, which is evidence of serine peptidases. These types of peptidase are generally

alkaline. If the optimum pH for these serine peptidases is close to pH 12, it is probable for CES to retain its activity at this pH level.

Optimum temperature of peptidase and temperature stability

Peptidase activity results for CES and PPPase at different temperatures (4-70°C) are shown in Fig. 5. The optimum temperature of the peptidase solutions was determined as approximately 58-60°C and an apparent decrease in activity was observed after this temperature. The optimum temperature of *B. cereus* metallopeptidase was determined as 37, 45, and 55°C by D'Costa *et al*. (2013), Sousa *et al*. (2007), and Manni *et al*. (2008), respectively. Lee *et al*. (2002) determined the optimum temperature of a keratinolytic metallopeptidase produced by *Bacillus* sp. SCB-3 as 40°C; on the other hand, metallopeptidases produced by *B. mojavensis* Roberts et al. (Amal & Abdelouahab 2017), *B. licheniformis* Weigmann (Mardina & Yusof 2016), *B. subtilis* (Ehrenberg) Cohn (Lauer *et al*. 2000, Rehman *et al*. 2017) and *B. polymyxa* B-17 Prazmowski (Sabirova *et al*. 2010) showed optimum activity at 70, 65, 55 and 50°C, respectively. *Bacillus* spp. G51 and *Bacillus* sp. APCMST-CS4 peptidases were determined to show optimum proteolytic activity at 60°C (Iglesias *et al*. 2017, Maruthiah *et al*. 2017). Cold-active metallopeptidase, which shows optimum enzyme activity at 20°C, was also purified from *Bacillus* sp. (Furhan *et al*. 2019). It is evident from the results of different studies that metallopeptidases produced by different strains of the same species can have different optimum temperatures.

The initial peptidase activity of CES and PPPase prior to the incubation applied for the stability tests was measured as 37.05 and 33.64 U/mL, respectively (Fig. 6). Results indicated that CES exhibited overall a higher stability profile compared with PPPase, which may imply that some of the impurities that were present in the crude supernatant had a stabilizing effect on the enzymes. Both enzyme fractions were inactivated immediately at 70°C, and at 60°C, which was the optimum temperature for peptidolytic activity of *B. amyloliquefaciens* FE-K1, 93.5 % of PPPase activity was maintained for the first 10 minutes and lost quickly after that. It may be advisable to apply a milder temperature than the optimum in using either fraction and carry out digestion at 50°C where the enzyme half-life would be approximately one hour. The results obtained are comparable to those of Manni *et al*. (2008) who also reported that 60 % of metallopeptidase activity remained after incubation at 50°C for 1 h, and of Sousa *et al*. (2007) who found 55 % activity remaining at the same temperature after 2 h of incubation. Maruthiah *et al*. (2017) reported that a more than 80 % activity of their serine metallopeptidase remained after 1.3 h of incubation within the temperature range between 40 and 60°C. Furhan *et al*. (2019) observed that more than half of the activity of cold-active metallopeptidase was retained between 20-30°C.

Effect of peptidase inhibitors on enzyme activity

The effect of peptidase inhibitors was investigated in the purification step for different fractions (Table 1). In this section, the effect of different concentrations of the inhibitors on enzyme activity was tested. The results are given in Table 3 as relative activity (%) according to the activity of inhibitor-free enzyme solutions. The activity of inhibitor-free CES and PPPase was 81.23 U/mL and 40.76 U/mL, respectively. Activity analysis was tested at optimum pH (7.5) and temperature (60°C). A large decrease in peptidase activities of CES and inactivation of PPPase were observed in the presence of O-FEN and EDTA, which are inhibitors of metallopeptidases. The Pearson correlation test results were given in Table 4. The decrease in activity was correlated with the concentration of O-FEN: a higher concentration of O-FEN resulted in a higher decrease in peptidase activity ($p < 0.05$). It was considered that an O-FEN concentration higher than 4 mM might completely inactivate the enzyme. It was determined that even the lowest concentration of EDTA completely inactivated PPPase, but the decrease in CES activity did not depend on EDTA concentration. Probably, in the presence of EDTA, even at the lowest concentration, all of the metallopeptidases were inactivated, and the remaining activity of 12 % could be due to another types of peptidases.

The enzyme solutions in our study are comprised mostly of metallopeptidases; however, they also contain other types of peptidases since there is a decrease in activity in the presence of peptidase inhibitors other than O-FEN and EDTA. The variation in the activity of other peptidases was not correlated to the increase in inhibitor concentrations, probably due to the low amount of other peptidases. A significant positive correlation was observed between PMSF concentration and peptidase activity of CES, but it may be due to the complex structure of the CES (Table 4).

Rehman *et al*. (2017) and Si *et al*. (2018) reported their enzyme as metallopeptidase after observing a considerable amount of decrease in the activity in the presence of EDTA. Manni *et al*. (2008) declared their peptidase as a metallopeptidase due to its complete inactivation at 20 mM of EDTA. Furthermore, they related the decrease in peptidase activity with increasing PMSF concentration to the presence of a serine group close to the active site of the enzyme. Sabirova *et al*. (2010) determined the remaining enzyme activity as 3.9 % and 0.1 % in the presence of 10 mM of EDTA and 10 mM of O-FEN, respectively.

Effect of some organic solvents and surfactants on peptidase activity

Enzymes are generally inactivated or denatured in the presence of organic solvents. However, being resistant to organic solvents makes the use of an enzyme in synthesis reactions possible. Peptidases are among the most valuable catalysts used in food, pharmaceutical, and detergent industries because they hydrolyse peptide bonds in aqueous environments and synthesize peptide bonds in microaqueous environments (Gupta 1992, Rahman *et al*. 2007). Additionally, peptidases used in detergent industry must be resistant to detergent supplements (Kumar & Takagi 1999).

Table 3. Effect of peptidase inhibitors on enzyme activity at different concentrations (relative activity, %).

Inhibitors	Concentration (µM)	CES	PPPase
PEP-A	5	91.28 ± 6.17	88.92 ± 1.84
	10	88.33 ± 4.47	81.53 ± 1.84
	20	89.97 ± 0.00	85.01 ± 0.61
E-64	10	90.41 ± 0.31	88.48 ± 1.23
	20	89.10 ± 5.55	75.45 ± 1.23
	40	95.42 ± 1.85	77.62 ± 0.00
PMSF	1	76.01 ± 1.54	88.27 ± 0.31
	2	79.39 ± 0.46	74.58 ± 0.00
	4	86.15 ± 2.62	87.40 ± 0.31
O-FEN	1	38.21 ± 0.16	33.38 ± 0.41
	2	26.51 ± 0.51	8.12 ± 0.28
	4	17.16 ± 0.09	1.93 ± 0.09
EDTA	1	12.04 ± 0.39	0.00 ± 0.00
	2	12.22 ± 0.14	0.00 ± 0.00
	4	13.41 ± 0.16	0.00 ± 0.00

PEP-A: Pepstatin A; E-64: Epoxysuccinyl-L-leucylamido-(4-guanidino) butane; PMSF: Phenylmethylsulphonyl fluoride; O-FEN: 1,10-phenanthroline; EDTA: Ethylenediaminetetraacetic acid

The peptidase activity was measured at optimum temperature (60°C) and pH (7.5) after the enzyme solution and inhibitors were mixed and kept at 37°C for 30 min.

Table 4. Correlation results between inhibitor concentration and relative peptidase activity.

Inhibitor	CES	PPPase
PEP-A	r = -0.096 p-value = 0.856	r = -0.338 p-value = 0.513
E-64	r = 0.648 p-value = 0.164	r = -0.640 p-value = 0.171
PMSF	r = 0.958 p-value = 0.003	r = 0.133 p-value = 0.802
O-FEN	r = -0.968 p-value = 0.002	r = -0.864 p-value = 0.026
EDTA	r = 0.936 p-value = 0.006	*

r: The Pearson correlation coefficient

r value closed to "1" or "-1" represents a positive or negative relationship, respectively.

The p-value of less than 0.05 indicates that the correlation is statistically significant.

*Peptidase activity was zero for all EDTA concentrations.

The effects of some organic solvents and surfactants on CES and PPPase are given in Table 5. It was observed that enzyme solutions were resistant to the applied organic solvents, and there was no substantial loss in enzyme activity. Furthermore, all of the applied organic solvents resulted in an increase in the enzyme activity of CES. Xylene and ethanol at higher concentrations, and acetone and acetonitrile at lower concentrations were more effective for increasing the peptidase activity. An increase in PPPase activity was only obtained in the presence of xylene and acetonitrile.

A 14 % increase in PPPase activity was obtained in the presence of 1 % acetonitrile (Table 5). Sousa *et al.* (2007) determined the increase as 3 % under the same conditions as in our study. A decrease in activity was observed in the presence of ethanol and acetone. It is noteworthy that more than 85 % of the activity was retained after incubation at 37°C for 30 min. However, *B. cereus* metallopeptidase retained 80 % of its initial activity after incubation at 60°C for 4 h in the presence of benzene, toluene, acetone, xylene and isopropanol (Doddapaneni *et al*. 2009). Wang *et al.* (2006) determined that the peptidase activity of *Bacillus* sp. TKU004 CES remaining after incubation at 4 and 25°C for 10 days was 65 %. In another study, it was shown that the activity decreased by 21 % and increased by 31 % with acetone and ethanol, respectively (Maruthiah *et al*. 2017). *Bacillus* sp. metallopeptidase was found stable in the presence of several organic solvents (40 %, v/v) after 1 h at 40°C (Qureshi *et al*. 2018).

Although the resistance of CES to detergent supplements was higher than that of PPPase, the results showed that neither enzyme solutions showed resistance to SDS. The peptidase activity of CES decreased to a level of 5 % depending on the concentration in the presence of SDS. Complete inactivation, on the other hand, was observed for PPPase. Approximately 80 % of the peptidase activity was retained in the presence of Triton X-100. It was observed that the activity increased by 8 % in the presence of 0.1 % Triton X-100; however, the activity decreased with increasing concentration. Sousa *et al.* (2007) determined similarly that *B. cereus* metallopeptidase was inactivated by SDS and retained most of its activity in the presence of Triton X-100. In contrast, *B. cereus* SV1 metallopeptidase retained 90 % and 35 % of its activity in the presence of 0.1 % and 1.0 % SDS, respectively (Manni *et al*. 2010). Rehman *et al.* (2017) determined an increase in peptidase activity in the presence of SDS, but a decrease with Triton X-100. Mardina & Yusof (2016) observed that *B. licheniformis* metallopeptidase was stable with 1 % Triton X-100, but the activity of the enzyme was increased by 82.7 % in the presence of SDS. Iglesias *et al.* (2017) determined a 2.9 % increase and an approximately 20 % decrease in the activity of *Bacillus* sp. G51 metallopeptidase in the presence of 1 % and 5 % Triton X-100, respectively.

A positive effect of Tween 20 and Tween 80 on peptidase activity was also observed (Table 5). The activity of CES was increased by 25 % and 29 % in the presence of 1 % Tween 20 and Tween 80, respectively. Tween 20 (0.1 %) caused a decrease in PPPase activity; however, stabilization was obtained at higher concentrations of Tween 20. Tian *et al.* (2019) observed a 14 % and 31 % increase in the enzyme activity with 1 % Tween 20 and Tween 80, respectively.

Effect of metal ions on enzyme activity

The effect of metal ions on peptidase activity is shown in Table 6. The activities of metal ion-free and 1:1 diluted CES and PPPase were 34.09 U/mL and 20.00 U/mL, respectively. The activity was tested at optimum

pH (7.5) and temperature (60°C). It was found that the peptidase activity was wholly inactivated by Hg^{+2}. It has been reported that even trace amounts of heavy metals inactivate most peptidases, and Hg^{+2} is an inhibitor of thermolysin, which is a well-known metallopeptidase (Bankus & Bond 2001). It was also observed that the metal ions Cu^{+2}, Ni^{+2}, and Fe^{+2} caused a substantial decrease in peptidase activity. Furthermore, the peptidase activity of PPPase was almost wholly inactivated in the presence of 5 mM Fe^{+3}. Bankus & Bond (2001) reported Fe^{+3} as an inhibitor of metallocarboxipeptidases. This information supports the idea that PPPase is a metallopeptidase-rich enzyme solution. Abdel-Naby *et al*. (2017) and Rehman *et al*. (2017) also observed a substantial inactivation in the enzyme activity in the presence of Hg^{+2}, Fe^{+3} and Cu^{+2}. In contrast to our study, Rehman *et al*. (2017) determined Ni^{+2} as a stimulator of *B. subtilis* KT004404 metallopeptidase activity.

It was determined in some studies that metallopeptidase activity increased by 7 % (D'Costa *et al*. 2013), 17 % (Sousa *et al*. 2007), 34 % (Abdel-Naby *et al*. 2017), 94 % (Manni *et al*. 2008), 200 % (Manni *et al*. 2010) and even 4120 % (Sharmila *et al*. 2018) in the presence of Ca^{+2} ions. It has been reported that Ca^{+2} ions stabilize thermolysin by preventing autolysis (Wu & Chen 2011). However, in our study, it was observed that Ca^{+2} ions caused a decrease in peptidase activity, and also that increasing the concentration of Ca^{+2} ions resulted in the precipitation of peptidase. Similarly, Furhan *et al*. (2019) determined that Ca^{+2} ions caused a 57 % decrease in the activity of cold-active metallopeptidase.

Among the metal ions, the most positive effect on peptidase activity was obtained with Mn^{+2} and K^{+1}. It was determined that 5 mM of K^{+1} and 5 mM of Mn^{+2} ions increased the enzyme activity of PPPase by 4 % and 6 %, respectively. In the presence of Mn^{+2} ions, a 178 % increase (Lee *et al*. 2016) and a 17 % decrease (Manni *et al*. 2008) in peptidase activity were determined.

Table 5. Effects of organic solvents and surfactants on enzyme activity at different concentrations (relative activity, %).

		Concentration (%)	CES	PPPase
Organic solvents	Xylene	1	86.25 ± 0.15	88.91 ± 0.67
		5	100.94 ± 2.86	97.88 ± 1.00
		20	102.81 ± 0.29	106.96 ± 3.51
	Acetone	1	110.62 ± 0.15	92.92 ± 1.34
		5	94.27 ± 0.59	93.04 ± 1.17
		20	103.02 ± 3.83	93.15 ± 1.00
	Ethanol	1	95.73 ± 0.59	86.78 ± 0.00
		5	111.46 ± 0.44	87.13 ± 2.84
		20	113.96 ± 0.74	85.72 ± 0.83
	Acetonitrile	1	122.60 ± 0.88	114.28 ± 3.17
		5	126.66 ± 3.39	105.31 ± 0.15
		20	62.09 ± 0.74	84.30 ± 0.15
Surfactants	SDS	0.1	14.86 ± 2.28	0.03 ± 0.00
		0.5	6.86 ± 1.48	0.00 ± 0.00
		1	5.64 ± 0.34	0.00 ± 0.00
	Triton X-100	0.1	108.55 ± 3.09	82.31 ± 0.24
		0.5	84.98 ± 2.02	67.07 ± 1.07
		1	81.66 ± 0.00	75.74 ± 1.19
	Tween 20	0.1	98.19 ± 0.00	79.28 ± 0.48
		0.5	116.63 ± 2.69	92.00 ± 2.03
		1	125.00 ± 9.41	100.67 ± 1.43
	Tween 80	0.1	122.62 ± 9.01	97.56 ± 0.12
		0.5	127.85 ± 8.06	106.74 ± 0.71
		1	129.08 ± 3.63	104.89 ± 1.67

The peptidase activity was measured at optimum temperature (60°C) and pH (7.5) after the enzyme solution and organic solvents or surfactants were mixed and kept at 37°C for 30 min.

Table 6. The effect of metal ions on enzyme activity (relative activity, %).

Metal ions	CES			PPPase		
	0.5 mM	2 mM	5 mM	0.5 mM	2 mM	5 mM
Ca^{+2}	81.48 ± 0.97	86.39 ± 1.98	*	87.63 ± 0.73	93.00 ± 4.53	87.36 ±0.36
Co^{+2}	71.71 ± 0.24	73.72 ± 3.17	*	96.22 ± 1.21	80.02 ± 7.62	74.80 ±0.38
Cu^{+2}	77.37 ± 0.48	42.81 ± 1.02	36.91 ± 0.33	58.95 ± 0.00	56.61 ± 3.03	34.49 ±0.88
Fe^{+2}	80.28 ± 4.12	69.31 ± 0.69	*	72.69 ± 0.97	25.96 ± 0.19	53.96 ±0.50
Fe^{+3}	70.51 ± 0.48	84.54 ± 8.71	*	81.45 ± 1.70	19.55 ± 5.09	0.19 ±0.13
Mg^{+2}	87.66 ± 1.45	95.16 ± 1.50	106.24±2.42	96.57 ± 0.73	86.22 ± 2.16	93.46 ±0.55
Hg^{+2}	4.32 ± 0.48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00
Mn^{+2}	86.28 ± 0.48	78.25 ± 0.73	*	92.96 ± 1.94	104.26±1.45	106.15±4.70
Zn^{+2}	68.79 ± 1.94	80.85 ± 2.65	*	90.90 ± 4.86	91.01 ± 0.63	80.69 ±1.88
Ni^{+2}	81.83 ± 0.97	32.47 ± 8.69	22.81 ± 1.80	65.65 ± 2.67	28.88 ± 1.20	14.30 ±0.48
K^{+1}	112.18 ± 0.24	92.54 ± 4.27	95.37±11.72	102.92 ± 0.97	100.34±9.60	104.42±2.71
Na^{+1}	89.71 ± 5.33	94.73 ± 0.77	112.63±1.54	94.85 ± 1.70	96.09±11.08	98.10 ±2.26

*Precipitation was observed when the enzyme was mixed with the metal ion (especially after incubation).
The activities of metal ion-free and 1:1 diluted CES and PPPase were 34.09 U/ml and 20.00 U/ml, respectively.
Activity analyses were tested at optimum pH (7.5) and temperature (60°C) after the enzyme solution and metal ions were mixed and kept at 37°C for 30 min.

Conclusions

Characterization of the PPPase from *B. amyloliquefaciens* FE-K1 reveals the enzyme as a neutral metallopeptidase compatible with some organic solvents and surfactants which are commonly used in detergents. Moreover, since the strain isolated from bread has previously been identified as a non-toxigenic organism, enzyme products obtained from the culturing of the strain may be used in the food industry. Using neutral and low thermotolerant enzymes have some advantages in food industry, because it is easier to control them during food processing. Future studies will focus on utilizing the *B. amyloliquefaciens* FE-K1 peptidase for the modification of gluten proteins in flour to reduce the gluten content of bread.

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Research Article

FIRST REPORT OF Cucumis melo endornavirus WITH A NEW HOST, THE GHERKIN (*Cucumis anguria* Linn.), IN TURKEY

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CmEV

Abstract: More than 50 viruses causing infection in members of the family Cucurbitaceae have been identified in the world so far. Because of the development of virus detection methods, new viruses are added to the known infectious cases list every day. One of the viruses recently identified is the Cucumis melo endornavirus (CmEV) which has been reported from different countries all over the world. However, no study for determination of CmEV has been done so far in Turkey. For the purpose of this study, 59 cucurbit plants showing virus and virus-like symptoms were collected from Manisa and İzmir provinces in Turkey. The samples were tested, for presence of CmEV, by reverse-transcriptase polymerase chain reaction and CmEV infections were detected in 47 samples, of which 44 were melon (*Cucumis melo* Linn.), and the remaining were gherkins (*C. anguria* Linn.). Among the infected samples, five samples (three were melon, and two were gherkin) were chosen for sequence analysis. After evaluating the sequence analysis results, it was shown that the Turkish isolates presented 93%-99% and 93%-98% identities at the nucleotide level and 94%-99% and 91%-98% identities at the amino acid level among each other and worldwide isolates, respectively. To the best of our knowledge, this is the first report of CmEV in gherkin as a new host both in Turkey and worldwide.

Özet: Dünyada gerçekleştirilen çalışmalar sonucunda kabakgil familyasındaki bitkileri enfekte eden 50'den fazla virüs varlığı tanımlanmıştır. Virüs tespit metotlarındaki gelişmelere bağlı olarak bu sayıya her geçen gün yenileri eklenmektedir. Son yıllarda tanılanan bu virüs hastalıklarından bir tanesi de Cucumis melo endornavirus (CmEV)'dır. CmEV'nin enfeksiyonu dünyanın farklı ülkelerinden bildirilmiştir. Ancak, ülkemizde şimdiye kadar CmEV varlığının belirlenmesine yönelik bir çalışma gerçekleştirilmemiştir. Bu amaçla, Manisa ve İzmir illerinden virüs ve virüs-benzeri belirti gösteren 59 kabakgil bitkisinden örnekler alınmıştır. Toplanan örnekler ters transkriptaz-polimeraz zincir reaksiyonu ile test edilmiştir. Gerçekleştirilen bu testler sonucunda 47 örnekte CmEV enfeksiyonu tespit edilmiştir. Enfekteli 47 örneğin 44 tanesi kavun (*Cucumis melo* Linn.), kalan 3 örnek ise acurdur (*C. anguria* Linn.). Enfekteli örnekler arasından, beş tanesi (üç tanesi kavun, iki tanesi acur) sekans analizleri için seçilmiştir. Gerçekleştirilen sekans analizleri sonucunda CmEV izolatları kendi aralarında nükleotit düzeyinde %93-99, amino asit düzeyinde ise %94-99 benzerlikler gösterdiği belirlenmiştir. Dünya izolatları ile yapılan benzerlik analizleri sonucunda ise nükleotit düzeyinde %93-98, amino asit düzeyinde ise %91-98 benzerlikler tespit edilmiştir. Gerçekleştirilen bu çalışma ile ülkemizde ilk kez CmEV enfeksiyonu kavun ve dünya için yeni bir konukçu kaydı olan acur bitkisinde tespit edilmiştir.

Introduction

Turkey is characterized with a wide range of ecological diversity which allows cultivation of different plant taxa one of which includes the cucurbit plants in the family Cucurbitacea (Çat *et al.* 2016). The currently known 825 species of this family are placed in 118 genera (Jeffrey 1980). The most important species of this family include melon (*Cucumis melo* Linn.), watermelon (*Citrullus lanatus* Thumb.), gherkin (*Cucumis anguria* Linn.), and zucchini/squash/pumpkin (*Cucurbita* spp.) all which can be cultivated in almost every region of the world and provide important economic inputs to their producers.

There are many viral diseases known to affect members of this important plant family (Zitter *et al.* 1996) by causing economic losses. New viruses are increasingly being added to the list of disease agents and they threaten the production of cucurbit plants. Some of these viruses are routinely studied, while others are not. Therefore, there is no reliable available information about the importance and prevalence of these viruses.

A number of viruses infecting cucurbit plants in Turkey were identified (Köklü & Yilmaz 2006, Ozaslan *et al*. 2006, Kamberoglu *et al*. 2016), among which the most prevalent viruses are the Watermelon mosaic virus, Cucumber mosaic virus, and the Zucchini yellow mosaic virus (Kaya & Erkan 2011, Keçe & Kamberoğlu 2016, Topkaya *et al*. 2019).

Cucumis melo endornavirus (CmEV) is one of the recently identified viral disease agent infecting the cucurbit plants. CmEV is a member of the endornavirus family that infects plants and fungi. The causal agent has (+)ssRNA genome containing nucleotides of around 15 kb (Valverde *et al*. 2019).

The presence of CMeV has so far been reported only from Ecuador, USA and Brazil (Quito-Avila *et al*. 2014, Sabanadzovic *et al*. 2016, da Costa *et al*. 2019). Based on a Japanese study, it was shown that dsRNA bands close to the CmEV genome were obtained from melon via dsRNA analysis (Fukuhara *et al*. 2006). CmEV sequences from South Korea are available in the GenBank. However, there has been no attempt in Turkey so far to detect the presence of CmEV infection. In the present study, field studies were performed on cucurbit plants grown in Manisa and İzmir provinces on Turkey and the presence of the CmEV as the causal agent was investigated.

Materials and Methods

Sampling and virus detection

Samples were collected in randomly selected fields in İzmir and Manisa provinces among cucurbit plants only showing the virus and virus-like symptoms (Fig. 1). The field studies were carried out from June to September in 2019. When similar symptoms were observed in the same field, only three samples were collected.

The presence of CmEV in the collected samples was determined by reverse-transcriptase polymerase chain reaction (RT-PCR) using a virus-specific primer pair. Before performing RT-PCR, total nucleic acid (TNA) isolation was performed by the cetyl trimethylammonium bromide (CTAB) method (Li *et al*. 2008). The resulting TNAs were checked by agarose gel electrophoresis and stored at -80 ºC until used.

To determine the presence of CmEV in the resulting TNAs, complementary DNAs (cDNAs) were initially synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific ™, USA). The presence of CmEV was then screened by PCR with the CmEV-primer pair (Table 1) specific to the partial polyprotein gene. PCR tests were performed according to conditions as indicated by Quito-Avila *et al*. (2014) using 2X EmeraldAmp® Max PCR Master Mix (Takara, Japan).

Fig. 1. Map showing the provincial borders of the two provinces (shaded in green) in the western part of Turkey where the field samplings were performed. The numbers in parentheses correspond to the sampling numbers in each province.

Sequence analysis

Five samples were chosen among the samples which were accepted to be infected with CmEV based on host species and the geographic origin, where they were obtained from. The resulting RT-PCR products were purified by EZ-10 Spin Column PCR Products (BioBasic, Canada) and sequenced bilaterally.

The assembled sequences were used to match with other world CmEV isolates from the GenBank (Table 2). Sequence identities of the isolates were determined with the sequence Demarcation Tool V. 1.2 (Muhire *et al*. 2014). The phylogenetic relationship was determined in CLC Main Work Bench V. 20 packet program (Qiagen, Canada) using the neighbor-joining method by applying Kimura 2-parameter with 1000 bootstrap replications.

Table 1. Primer pair used in RT-PCR for determination of CmEV infection in the collected samples.

Primer code	Primer sequence (5'-3')	Sense	Fragment size	Reference
CmEVF	GGTGGAATATGGGTTGATGCTAG	Forward	413 bp	Quito-Avila *et al*. (2014)
CmEVR	CGTCGTGATGGACATCAACTCTAC	Reverse		

Table 2. CmEV isolates used in molecular characterization studies

Isolate Code	Accession Number	Host	Origin	Reference
SJ1	KX641269	Korean melon	South Korea	Baek *et al.* (2016)
CL-01	NC_029064	Melon	USA	Sabanadzovic *et al.* (2016)
BRA/TO-74/2010	MH365459	Not known*	Brazil	da Costa *et al.* (2019)
BRA/TO-23/2014	MH365458	Not known*	Brazil	da Costa *et al.* (2019)
MAN2	MN985120	Melon	Turkey	This study
MAN22	MN985121	Gherkin	Turkey	This study
MAN25	MN985122	Melon	Turkey	This study
IZM7	MN985123	Gherkin	Turkey	This study
IZM36	MN985124	Melon	Turkey	This study

*found in human stool samples

Results

A total of 59 cucurbit plants with viruses and virus-like symptoms were collected. Forty-nine of these were melon, seven were pumpkins, and three were gherkins. CmEV was detected in 47 of the samples (Table 3).

With the exception of five collected melon samples, nearly all were found to be infected with CmEV. Three CmEV infections were detected in gherkin plants. CmEV infection was detected in none of the pumpkin plants.

As a result of the sequence analysis performed, it was determined that Turkish CmEV isolates showed 93%-99% and 94%-99% identities with each other at the nucleotide and amino acid levels, respectively. In multiple sequence alignments with world CmEV isolates, it was observed that Turkish CmEV isolates showed 93%-98% and 91%-98% identities at the nucleotide and amino acids levels, respectively. The highest nucleotide identity rate between the Turkish and world CmEV isolates was found to be 98% between MAN25 with BRA/TO-74/2010 and BRA/TO-23/2014 isolates, while the least nucleotide identity was found to be 93% between MAN22 with CL01 and SJ1 isolates (Fig. 2).

Based on the phylogenetic analysis, all of the Turkish CmEV isolates, except MAN25 and IZM36, were closely related and formed a separate clade. While the world isolates form a clade with MAN25, IZM36 was found as a separate clade that consists of only itself (Fig. 3).

Table 3. The numerical distribution of the sampled cucurbits in each province. The numbers of infected samples were also given for each different plant.

Cucurbit Species	Province		Total Number of Infected and Collected Samples
	Manisa	İzmir	
	Number of Infected/Collected Samples	Number of Infected/Collected Samples	
Melon	24/24	20/25	44/49
Pumpkin	0/1	0/6	0/7
Gherkin	2/2	1/1	3/3
Total Number of Infected/Collected Samples	26/27	21/32	47/59

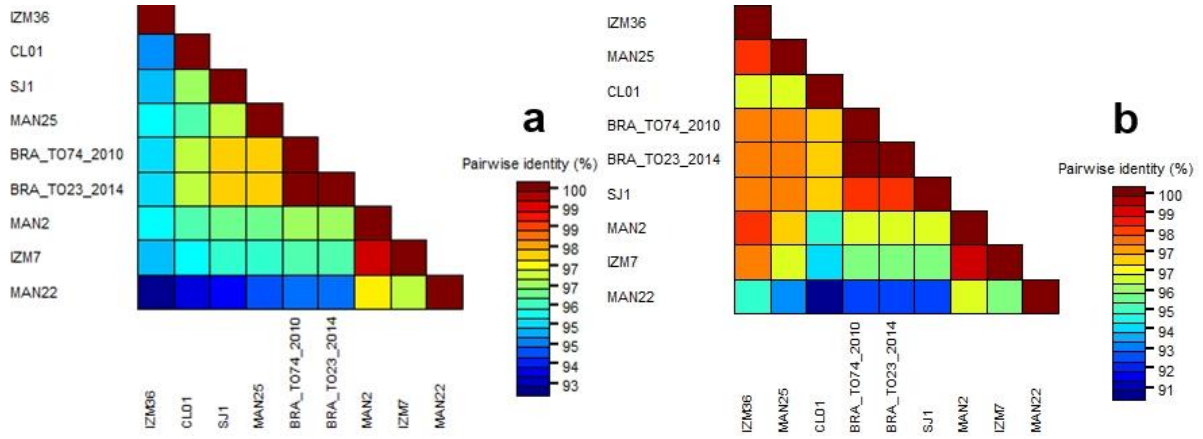

Fig. 2. Identity matrix of Turkish and world CmEV. The colored identity matrix was generated by using partial polyprotein gene region of CmEV based on nucleotide (a) and amino acid (b) sequences.

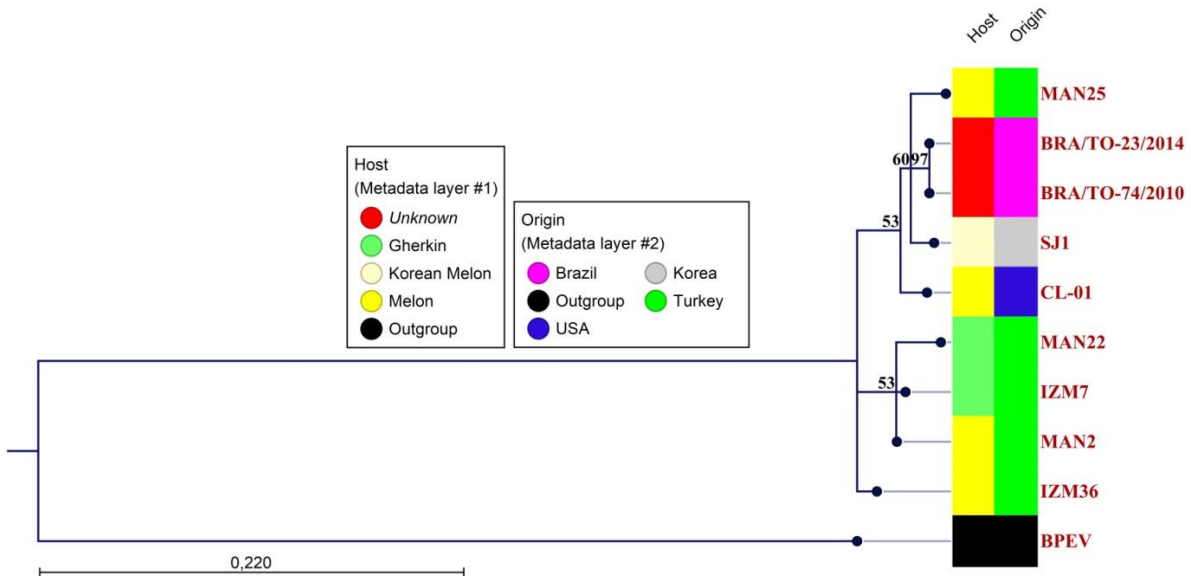

Fig. 3. Phylogenetic relationship of Turkish CmEV isolates based on the partial polyprotein gene region. The phylogenetic tree was generated by neighbor-joining method applying Kimura 80 parameters with 1000 bootstrap replications. The bootstrap threshold was implemented at 50%. Bell pepper endornavirus (BPEV) was used as the outgroup.

Discussion

Although the presence of CmEV from different countries has been reported, only one study (Quito-Avila *et al*. 2014) about the prevalence of the causal agent comes to the forefront. As a result of this study conducted in cucurbit production fields in Ecuador, it was reported that 95% of melon plants with and without virus-like symptoms were infected with CmEV. No CmEV infection was detected in watermelon and cucumber plants (Quito-Avila *et al*. 2014). In this context, the results obtained in the present study were found to be in parallel with the results of Quito-Avila *et al*. (2014).

It is known that endornaviruses infect plants and fungi. There are many economically important crops that are infected with these viruses. Some of these crops are rice, barley, pepper, and common bean (Wakarchuk & Hamilton 1985, Zabalgogeazcoa & Gildow 1992, Fukuhara *et al*. 1993, Okada *et al*. 2011). In a recent study from the United States (US), endornaviruses were detected in non-cultivated plant species (Herschlag *et al*. 2019). In addition to the known host range of endornaviruses, gherkin was found to be a new host for CmEV as the result of this study.

In a study carried out in the US, it was reported that CmEV populations had 10% and 6% genetic variation with each other at the nucleotide and amino acid levels, respectively (Sabanadzovic *et al*. 2016). In this context, the identity rates obtained in this study showed great parallelism with that study.

It was determined that the isolates did not show phylogenetic distribution according to their geographic origin and host (Fig. 3). However, it is considered necessary to perform analyses using a much larger number of isolates with larger sequences for more reliable results.

As a result of the recent worldwide studies, the host range of endornaviruses has been expanded. However, the impact of these viruses on crop yields or growth parameters is still unclear (Escalante *et al*. 2016, Fukuhara 2019), especially in cases in which the CmEV infected plant is co-infected with severe viruses. To the best of our knowledge, this is the first report of CmEV with gherkin (*C. anguria* Linn.) as a new host both in Turkey and worldwide. Moreover, it is thought that the virus may present a more extensive worldwide distribution than previously reported.

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Başlık: İngilizce olarak Kısa ve açıklayıcı olmalı, büyük harfle ve ortalanarak yazılmalıdır.

Özet ve Anahtar kelimeler: Türkçe ve İngilizce özet 250 kelimeyi geçmemelidir. Özetin altına küçük harflerle anahtar kelimeler ibaresi yazılmalı ve yanına anahtar kelimeler virgül konularak sıralanmalıdır. Anahtar kelimeler, zorunlu olmadıkça başlıktakilerin tekrarı olmamalıdır. İngilizce özet koyu harflerle "Abstract" sözcüğü ile başlamalı ve başlık, İngilizce özetin üstüne büyük harflerle ortalanarak yazılmalıdır. Yazıdaki ana başlıklar ve varsa alt başlıklara **numara verilmemelidir.**

Giriş: Çalışmanın amacı ve geçmişte yapılan çalışmalar bu kısımda belirtilmelidir. Yazıda SI (Systeme International) birimleri ve kısaltmaları kullanılmalıdır. Diğer kısaltmalar kullanıldığında, metinde ilk geçtiği yerde 1 kez açıklanmalıdır. Kısaltma yapılmış birimlerin sonuna nokta konmamalıdır (45 m mesafe tespit edilmiştir). Kısaltma cümle sonunda ise nokta konmalıdır (… tespit edilen mesafe 45 m. Dolayısıyla…).

Materyal ve Metod: Eğer çalışma deneysel ise kullanılan deneysel yöntemler detaylı ve açıklayıcı bir biçimde verilmelidir. Yazıda kullanılan metod/metodlar, başkaları tarafından tekrarlanabilecek şekilde açıklayıcı olmalıdır. Fakat kullanılan deneysel yöntem herkes tarafından bilinen bir yöntem ise ayrıntılı

açıklamaya gerek olmayıp sadece yöntemin adı verilmeli veya yöntemin ilk kullanıldığı çalışmaya atıf yapılmalıdır.

Sonuçlar: Bu bölümde elde edilen sonuçlar verilmeli, yorum yapılmamalıdır**.** Sonuçlar gerekirse tablo, şekil ve grafiklerle de desteklenerek açıklanabilir.

Tartışma: Sonuçlar mutlaka tartışılmalı fakat gereksiz tekrarlardan kaçınılmalıdır. Bu kısımda, literatür bilgileri vermekten çok, çalışmanın sonuçlarına yoğunlaşmalı, sonuçların daha önce yapılmış araştırmalarla benzerlik ve farklılıkları verilmeli, bunların muhtemel nedenleri tartışılmalıdır. Bu bölümde, elde edilen sonuçların bilime katkısı ve önemine de mümkün olduğu kadar yer verilmelidir.

Teşekkür: Mümkün olduğunca kısa olmalıdır. Teşekkür, genellikle çalışmaya maddi destek sağlayan kurumlara, kişilere veya yazı yayına gönderilmeden önce inceleyip önerilerde bulunan uzmanlara yapılır. Teşekkür bölümü kaynaklardan önce ve ayrı bir başlık altında yapılır.

Kaynaklar: Yayınlanmamış bilgiler kaynak olarak verilmemelidir (*Yayınlanmamış kaynaklara örnekler: Hazırlanmakta olan veya yayına gönderilen yazılar, yayınlanmamış bilgiler veya gözlemler, kişilerle görüşülerek elde edilen bilgiler, raporlar, ders notları, seminerler gibi*). Ancak, tamamlanmış ve jüriden geçmiş tezler ve DOI numarası olan yazılar kaynak olarak verilebilir. Kaynaklar, yazı sonunda alfabetik sırada (yazarların soyadlarına göre) sıra numarası ile belirtilerek verilmelidir.

Yazıların ve kitapların referans olarak veriliş şekilleri aşağıdaki gibidir:

Makale: Yazarın soyadı, adının baş harfi, basıldığı yıl. Makalenin başlığı, *derginin adı*, cilt numarası, sayı, sayfa numarası. Dergi adı italik yazılır.

Örnek:

Tek yazarlı Makale için

Soyadı, A. Yıl. Makalenin adı. (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin açık ve tam adı*, Cilt(Sayı)**:** Sayfa aralığı.

Kıvan, M. 1998. *Eurygaster integriceps* Put. (Heteroptera: Scuteleridae)'nin yumurta parazitoiti *Trissolcus semistriatus* Nees (Hymenoptera: Scelionidae)'un biyolojisi üzerinde araştırmalar*. Türkiye Entomoloji Dergisi*, 22(4): 243-257.

İki ya da daha çok yazarlı makale için

Soyadı1, A1. & Soyadı2**,** A2. Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt(Sayı): Sayfa aralığı.

Lodos, N. & Önder, F. 1979. Controbution to the study on the Turkish Pentatomoidea (Heteroptera) IV. Family: Acanthasomatidae Stal 1864. *Türkiye Bitki Koruma Dergisi*, 3(3): 139-160.

Soyadı1, A1., Soyadı2, A2. & Soyadı3, A3**.** Yıl. Makalenin adı**.** (Sözcüklerin ilk harfi küçük). *Yayınlandığı derginin tam adı*, Cilt (Sayı): Sayfa aralığı.

Önder, F., Ünal, A. & Ünal, E. 1981. Heteroptera fauna collected by light traps in some districts of Northwestern part of Anatolia. *Türkiye Bitki Koruma Dergisi***,** 5(3): 151-169.

Kitap: Yazarın soyadı, adının baş harfi, basıldığı yıl. Kitabın adı (varsa derleyen veya çeviren ya da editör), cilt numarası, baskı numarası, basımevi, basıldığı şehir,toplam sayfa sayısı.

Örnek:

Soyadı, A., Yıl. *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Önder F., Karsavuran, Y., Tezcan, S. & Fent, M. 2006. *Türkiye Heteroptera (Insecta) Kataloğu*. Meta Basım Matbaacılık, İzmir, 164 s.

Lodos, N., Önder, F., Pehlivan, E., Atalay, R., Erkin, E., Karsavuran, Y., Tezcan, S. & Aksoy, S. 1999. Faunistic *Studies on Lygaeidae (Heteroptera) of Western Black Sea, Central Anatolia and Mediterranean Regions of Turkey.* Ege University, İzmir, ix + 58 pp.

Kitapta Bölüm: Yazarın soyadı, adının baş harfi basıldığı yıl. Bölüm adı, sayfa numaraları. Parantez içinde: Kitabın editörü/editörleri, *kitabın adı*, yayınlayan şirket veya kurum, yayınlandığı yer, toplam sayfa sayısı.

Örnek:

Soyadı, A., Yıl. Bölüm adı, sayfa aralığı. In: (editör/editörler). *Kitabın adı*. (Sözcüklerin ilk harfi büyük, italik). Basımevi, basıldığı şehir, toplam sayfa sayısı s./pp.

Jansson, A. 1995. Family Corixidae Leach, 1815—The water boatmen. Pp. 26–56. In: Aukema, B. & Rieger, Ch. (eds) Catalogue of the Heteroptera of the Palaearctic Region. Vol. 1. Enicocephalomorpha, Dipsocoromorpha, Nepomorpha, Gerromorpha and Leptopodomorpha. The Netherlands Entomological Society, Amsterdam, xxvi + 222 pp.

Kongre, Sempozyum: Yazarlar, Yıl. "Bildirinin adı (Sözcüklerin ilk harfi küçük), sayfa aralığı". Kongre/Sempozyum Adı, Tarihi (gün aralığı ve ay), Yayınlayan Kurum, Yayınlanma Yeri.

Örnek:

Bracko, G., Kiran, K., & Karaman, C. 2015. The ant fauna of Greek Thrace, 33-34. Paper presented at the 6th Central European Workshop of Myrmecology, 24-27 July, Debrecen-Hungary.

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Soyadı**,** A. Yıl. Çalışmanın adı. (Sözcüklerin ilk harfi küçük) (web sayfası) http://www.....) (Date accessed: 12.08.2009).

Hatch, S., 2001. Studentsperception of online education. Multimedia CBT Systems. (Web page: http://www.scu.edu.au/schools/sawd/moconf/papers2001/hatch.pdf) (Date accessed: 12.08.2009).

Kaynaklara metin içinde numara verilmemeli ve aşağıdaki örneklerde olduğu gibi belirtilmelidir.

Örnekler:
... x maddesi atmosferde kirliliğe neden olmaktadır (Landen 2002). Landen (2002) x maddesinin atmosferde kirliliğe neden olduğunu belirtmiştir. İki yazarlı bir çalışma kaynak olarak verilecekse, (Landen & Bruce 2002) veya Landen & Bruce (2002)'ye göre. ... şeklinde olmuştur; diye verilmelidir. Üç veya daha fazla yazar söz konusu ise, (Landen *et al.* 2002) veya Landen *et al.* (2002)'ye göre .... olduğu gösterilmiştir; diye yazılmalıdır.

Şekil ve Tablolar: Tablo dışında kalan fotoğraf, resim, çizim ve grafik gibi göstermeler "Şekil" olarak verilmelidir. Resim, şekil ve grafikler, net ve ofset baskı tekniğine uygun olmalıdır. Her tablo ve şeklin metin içindeki yerlerine konmalıdır. Tüm tablo ve şekiller yazı boyunca sırayla numaralandırılmalı (Tablo 1., Şekil. 1), başlık ve açıklamalar içermelidir. Şekillerin sıra numaraları ve başlıkları, alta, tabloların ki ise üstlerine yazılır.

Şekiller (tablo dışında kalan fotoğraf, resim, çizim ve grafik gibi) tek tek dosyalar halinde en az **300 dpi** çözünürlükte ve **tif** dosyası olarak şekil numaraları dosya isminde belirtilmiş şekilde ayrıca sisteme ek dosya olarak yüklenmelidir.

Sunulan yazılar, öncelikle Dergi Yayın Kurulu tarafından ön incelemeye tabii tutulur. **Dergi Yayın Kurulu, yayınlanabilecek nitelikte bulmadığı veya yazım kurallarına uygun hazırlanmayan yazıları hakemlere göndermeden red kararı verme hakkına sahiptir.** Değerlendirmeye alınabilecek olan yazılar, incelenmek üzere iki ayrı hakeme gönderilir. Dergi Yayın Kurulu, hakem raporlarını dikkate alarak yazıların yayınlanmak üzere kabul edilip edilmemesine karar verir.

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