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Investigation of beta-lactoglobulin derived bioactive peptides against SARS-CoV-2 (COVID-19): *In silico* analysis

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ABSTRACT

The coronavirus disease of 2019 (COVID-19) outbreak caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which started in late 2019 in Wuhan, China spread to the whole world in a short period of time, and thousands of people have died due to this epidemic. Although scientists have been searching for methods to manage SARS-CoV-2, there is no specific medication against COVID-19 as of yet. Two main approaches should be followed in the treatment of SARS-CoV-2; one of which is to neutralize the virus, and the other is to inhibit the host cell membrane receptors, where SARS-CoV-2 will bind. In this study, peptides derived from beta-lactoglobulin, which inactivates both the virus and its receptors in the host cell, were identified using computer-based *in silico* analysis. The beta-lactoglobulin derived peptides used in this study were obtained by the treatment of goat milk whey fraction with trypsin. The structure of the peptides was characterized by the liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS), and six beta-lactoglobulin derived peptides were selected as candidate peptides. Subsequently, the effects of peptides on SARS-CoV-2 and host cells were identified using virtual screening. According to the results of this *in silico* analysis, Ala-Leu-Pro-Met-His-Ile-Arg (ALMPHIR) and Ile-Pro-Ala-Val-Phe-Lys (IPAVFK) peptides were evaluated as potential candidates to be used in the treatment of SARS-CoV-2 after the future in vitro and in vivo studies.

1. Introduction

Viral diseases are still serious threats to public health all over the world. The major viral outbreaks (severe acute respiratory syndrome coronavirus [SARS-CoV], influenza A virus subtype [H1N1], and the Middle East Respiratory Syndrome coronavirus [MERS-CoV]) occurring in the last 20 years have caused serious deaths and unfortunately, the treatment is still unclear (Bradley and Bryan, 2019). World Health Organization (WHO) declared coronavirus disease of 2019 COVID-19 infection, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a pandemic on March 11, 2020 (WHO, 2020). To date, neither specific therapy nor a vaccine has been invented against COVID-19. The current management of COVID-19 is aimed to prevent and control the infection via empirical treatment methods and supportive care.

Once the structure of SARS-CoV-2 has been identified (Kong et al., 2020), researchers have focused on SARS-CoV-2 spike proteins and its main protease using *in silico* or experimental methods for neutralizing the virus or inhibiting its cellular entrance. The spike protein (S) of SARS-CoV-2, a trimeric transmembrane glycoprotein, recognizes the host cell and mediates its entry. It contains a receptor-binding domain (S1) and a domain that mediates the integration of the virus and host cell membranes (S2). For SARS-CoV-2 to enter cells, host cell proteases, furin, a calcium-dependent serine endopeptidase found in the host lung cell membrane, affects the S1/S2 cleavage site of the SARS-CoV-2 (Coutard et al., 2020). Due to this interaction, cleavage of S protein leaves the S2 domain exposed, and the transmission of SARS-Cov-2 into the cells is further facilitated (Xia et al., 2020). Accordingly, furin protease could be a promising approach against this infection. However, the main protease is one of the best-investigated drug targets against SARS-CoV-2. It is a homodimer that forms two active sites. Folding of the main protease is similar to serine proteases. However, a cysteine amino acid and a nearby histidine have protein-cleaving activity, and there is an extra domain that stabilizes its dimeric form. Thus, a peptide-like

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inhibitor binding to its active site can mediate the inhibition of virus replication (Kumar et al., 2017; Liu et al., 2005; Chen et al., 2006).

Another strategy to treat COVID-19 infection is the inhibition of entry of SARS-CoV-2 into the host cell via the angiotensin-converting enzyme (ACE) 2 receptors (Hoffmann et al., 2020). ACE 2 enzyme, discovered about 20 years ago, has been shown to increase Angiotensin (1–7) levels (Keidar et al., 2007; Donoghue et al., 2000). ACE 2 and ACE enzymes are biochemically similar and share similar protein sequences, but their substrates are different. Angiotensin 1 converts to angiotensin 1-9 by ACE2 and then to angiotensin 1-7 by ACE, while angiotensin II converts to angiotensin 1-7 by ACE2 enzyme. Angiotensin 1-7 is a biologically active metabolite that has opposite effects against angiotensin II and counteracts the opposite effects of angiotensin II in renin–angiotensin–aldosterone system (Santos et al., 2018). Angiotensin 1-7 provides cardioprotection through the increase in endothelial nitric oxide synthase (e-NOS) level, vasodilation, and anti-inflammatory effects.

Furthermore, recently, based on a docked complex model of COVID-19 spike glycoprotein and dipeptidyl peptidase-4 (DPP-4/also known CD26 or adenosine deaminase complexing protein 2), the importance of interactions between COVID-19 spike glycoproteins and host cell DPP-4 has been revealed (Vankadari and Wilce, 2020). This suggests the potential role of DPP-4 inhibitors by reducing the entry of SARS-CoV-2 into the cell. DPP-4 is a type II transmembrane protein (Tanaka et al., 1992) and removes dipeptides especially from Pro or Ala located at the penultimate position (Yoshimoto et al., 1978). DPP-4 inhibitors (such as sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin) increase insulin secretion, and reduce glucagon secretion via their effect on incretin hormone activity. They are clinically used in the management of diabetes (Holst and Deacon, 1998), and they are also known to be involved in inflammatory and infectious pathologic conditions (Klemann et al., 2016).

After the SARS-CoV-2 pandemic has appeared and the virus structure has been determined, new potential drugs that can interact with ACE2-related genes or prevent the virus from entering the host cell have been studied by *in silico* analysis (Cava et al., 2020). Peptide-like inhibitors, which are used in the management of various diseases, could also be potential agents against COVID-19. Pant et al. (2020) evaluated 300 peptide-like structures and determined sixty-six FDA-approved drugs by using a medication re-purposing approach (such as lopinavir, ritonavir, darunavir) against COVID-19 *in silico*. Another *in silico* study investigated an α-ketoamide inhibitor, a peptide-like inhibitor against the crystal structure of SARS-CoV-2 main protease and its complex (Zhang et al., 2020).

Milk proteins are composed of approximately 80% caseins and 20% whey proteins. The hydrolysis of these fractions yields various peptides. Although there are many studies related to the health benefits of casein-based peptides, the peptides of whey proteins have not fully been characterized yet. Whey bioactive peptides are beneficial to the cardiovascular system through in vitro ACE-inhibitory activity and in vivo antihypertensive effects. Furthermore, a DPP-4 inhibitor derived from goat milk casein has been shown to have an effect on the nervous system, immune system, and endocrine system (Sharma et al., 2017; Kitts and Weiler, 2003; Möller et al., 2008; Atanasova and Ivanova 2010; Zhang et al., 2015; Nongonierma and FitzGerald, 2015; Pihlanto-Leppälä, 2000).

Accordingly, in this study, the possible inhibitory effects of beta-lactoglobulin derived bioactive peptides on the spike protein and main protease of SARS-CoV-2, and inhibitory effects on ACE, furin, and DPP-4 enzymes were analyzed using *in silico* approaches (Fig. 1).

2. Materials and methods

2.1. Materials

Goat milk samples were obtained from Saanen-Maltız crossbred goats of the same age that were grown at a private farm in the Marmara Region of Turkey. Goat milk was kept at +4 °C after morning milking.

2.2. Preparation of the peptides from the whey fraction

The milk was defatted by centrifugation at 3000×*g* for 15 min at 4 °C followed by precipitation of caseins by adjusting the medium pH to 4.6 using 1 M HCl. Subsequently, the precipitated caseins were removed by centrifugation at 19,000×*g* for 1 h at 4 °C, and the resulting whey fraction was collected in the form of supernatant. Trypsin was used to digest the whey fraction. Whey fraction proteins were mixed with trypsin at 37 °C in 20:1 (wt/wt) ratio at pH 8. After digestion, each sample was heated at 98 °C for 10 min to inactivate the protease (Abubakar et al., 1998).

2.3. LC-Q-TOF-MS analysis

For protein extraction, the trypsin digested whey fraction was mixed with Universal Protein Extraction (UPX) Kit (Expedeon-44101) and protease inhibitor cocktail (Thermo Sci.-87785). Samples were sonicated at 200 g and then boiled at 95 °C for 10 min. After the boiling procedure, samples were centrifuged for 10 min at 20,000 g. Peptide

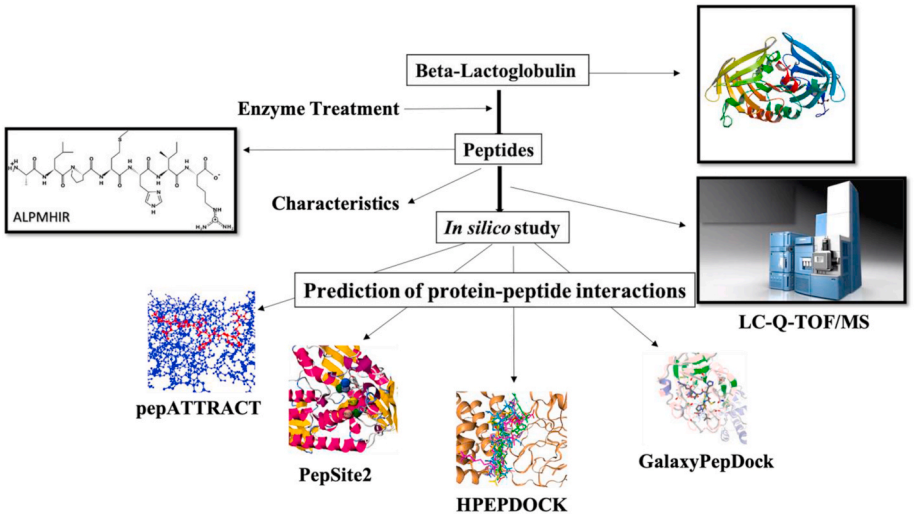

Fig. 1. Graphical abstract.

production was performed utilizing FASP Protein Digestion Kit (Expedeon-44250) and trypsin treatments (Pierce-90057) on the supernatant. Samples were diluted to 200 ng/μl with 0.1% formic acid. Detector and calibration settings were made with the MassLynx program (V4.1-Waters) that is specific to Xevo G2-XS Q-TOF-MS (Waters Corp, Milford, MA, USA) device where the analysis was carried out. The tryptic peptides were fractionated with an acetonitrile gradient (5–35%) in HSS T3 (Waters-186008818) column and analyzed by mass spectrometry upon electrospray ionization. Peptide data was identified in an m/z range of 50–1950. MS analysis was performed for 0.7 s and the data was collected from the entire peptide. UniProt protein database and Progenesis software were used for peptide identification.

2.4. Determination the roles of obtained peptides by BIOPEP-UWM and PepSite 2

The BIOPEP-UWM database (available at http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz et al., 2008) was used to predict the relevant inhibitory activities of the obtained peptides identified from LC–Q-TOF-MS analysis on the ACE and DPP-4 enzymes, the receptors to which SARS-CoV-2 binds on the cell surface. IPP (Isoleucine-Proline-Proline) peptide was used as a positive control (Watanabe et al., 2015) for inhibition of ACE and IPI (Isoleucine-Proline-Isoleucine; Diprotin A) peptide was used as a positive control (Song et al., 2017) for inhibition of DPP-4.

PepSite2 available at http://pepsite2.russelllab.org) (Trabuco et al., 2012) was used to predict the peptide-binding sites of *in silico* identified ACE and DPP-4 inhibitor peptides to ACE and DPP-4 enzymes.

ToxinPred (available at https://webs.iiitd.edu.in/raghava/toxinpred/index.html) (Gupta et al., 2013) was used to assess characteristic properties of beta-lactoglobulin derived peptides and predict its toxicity.

2.5. Molecular docking of beta-lactoglobulin derived peptides and the SARS-CoV-2 main protease and spike protein and host cell furin, ACE, DPP-4

The beta-lactoglobulin derived peptides were docked with SARS-CoV2 proteases (PDB:6LU7 and PDB:6M03), spike proteins (PDB:6VSB and PDB:6VXX) and host cell furin (PDB:1P8J), angiotensin-converting enzyme (ACE, PDB: 2YDM), and dipeptidyl peptidase-4 (DPP-4, PDB: 3WQH) receptors using HPEPDOCK (available at http://huanglab.phys.hust.edu.cn/hpepdock/) (Zhou et al., 2018), pepATTRACT (available at https://bioserv.rpbs.univ-paris-diderot.fr/services/pepATTRACT/) (Schindler et al., 2015) and GalaxyPepDock (available at http://galaxy.seoklab.org/pepdock) (Lee et al., 2015) servers. The HPEPDOCK is a server that investigates the protein-peptide docking based on the hierarchical algorithm. The Galaxy webserver was also used for confirming the output of the HPEPDOCK server. These docking scores in the HPEPDOCK server reflect the prediction of protein-ligand binding affinity by free energy in kcal/mol units (Zhou et al., 2018; Tao et al., 2020). GalaxyPepDock gives the similarity-based docking results by running energy-based optimization that allows structural flexibility. pepATTRACT is another peptide docking server that globally searches the entire protein surface, identifies the binding site, and predicts the bound peptide conformation.

3. Results

3.1. Peptides

Six beta-lactoglobulin (BLG) derived peptides (i.e., ALPMHIR (Ala-Leu-Pro-Met-His-Ile-Arg; $BLG^{160-166}$), GLDIQK(Gly-Leu-Asp-Ile-Gln-Lys; BLG^{27-32}), TPEVDK(Thr-Pro-Glu-Val-Asp-Lys; $BLG^{143-148}$), IPAVFK(Ile-Pro-Ala-Val-Phe-Lys; BLG^{96-101}), EALEK(Glu-Ala-Leu-Glu-Lys; $BLG^{149-153}$), and IIAEK(Ile-Ile-Ala-Glu-Lys; BLG^{89-93})) were characterized from the goat milk whey fraction. The characteristics of these beta-lactoglobulin derived peptides were presented in Table 1. MS spectrum that corresponds to the ionization of ALPMHIR, IPAVFK, and GLDIQK peptides was demonstrated in Fig. 2.

3.2. ACE and DPP-4 inhibitory activities of the beta-lactoglobulin derived peptides

The ACE and DPP-4 inhibitory activity scores of the beta-lactoglobulin derived peptides are presented in Table 2. According to the data obtained using the BIOPEP server, the frequency of a peptide in a protein sequence and potential biological activity of the peptide demonstrates the potential of the peptide to become a bioactive peptide.

Although all 6 peptides have been found to demonstrate both ACE and DPP-4 inhibitory activity; IPAVFK, IIAEK, and ALPMHIR displayed the higher ACE and DPP-4 inhibitory activities when compared to the other three peptides (Table 2).

3.3. Interaction mechanisms of ACE and DPP-4 enzymes with beta-lactoglobulin derived peptides

The amino acids of the beta-lactoglobulin derived peptides that potentially could interact with ACE (PDB:2YDM) and DPP-4 (3WQH) were listed in Table 3 and Table 4. Tables 3 and 4 also present the binding points of each peptide to the ACE and DPP-4 enzymes, respectively.

For ACE and DPP-4 inhibition, 3 different peptides display the best protein-peptide interaction (considering *p* values and potentially bound amino acids) were presented. Binding potential of ALPMHIR ($P < 0.0001$), IPAVFK ($P < 0.001$), IIAEK ($P < 0.001$), and TPEVDK ($P < 0.001$) peptides to the ACE enzyme was found to be statistically significant according to data obtained using the PepSite2 database (Table 3). ALPMHIR, IPAVFK, IIAEK, and TEPVDK appear to interact with all the amino acids that IPP bind on ACE enzymes. Furthermore, the ALPMHIR, IPAVFK, and TEPVDK peptides appeared to bind other than these amino acids that IPP binds on the ACE (Table 3).

Binding potential of ALPMHIR ($P < 0.001$), IPAVFK ($P < 0.05$), and TPEVDK ($P < 0.01$) peptides to the DPP-4 enzyme was also found to be statistically significant according to data obtained using the PepSite2 database (Table 4). As a result of protein-peptide interactions obtained concerning the DPP-4 inhibitor IPI peptide, ALPMHIR peptide was observed to bind to the same amino acids as the IPI peptide (Table 4). IPAVFK and TEPVDK peptides appear to bind all amino acids that IPI bind. However, these peptides bind to 4 different amino acids on DPP-4, where IPI did not bind on DPP-4 (Table 4).

3.4. Docking scores

3.4.1. HPEPDOCK

Table 5 presents the docking scores based on the minimum energy of the beta-lactoglobulin derived peptides and main protease (apo and holo forms) and spike protein of SARS-CoV-2 and host cell furin analysis with the HPEPDOCK.

According to Table 5, among the 6 peptides obtained from the beta-lactoglobulin fraction, the best docking score value was obtained from ALPMHIR (Fig. 3). The second-best score was obtained with IPAVFK.

3.4.2. pepATTRACT

According to the data obtained from the pepATTRACT program, the peptide molecules with the lowest binding energy level were ALPMHIR, IPAVFK, and GLDIQK. All beta-lactoglobulin derived peptides had lesser binding energy levels than that obtained from remdesivir and hydroxychloroquine. The interfacial propensity of peptides to interact with the main protease of SARS-CoV-12 and the energy values of these interactions were demonstrated in Table 6.

Table 1
Characteristics of the beta-lactoglobulin derived peptides.

Peptide Sequence	Prediction	Hydrophobicity	Steric hindrance	Hydropathicity	Hydrophilicity	Net Hydrogen	Charge	pI	Mol wt
ALPMHIR	Non-toxin	−0.07	0.51	0.39	−0.41	0.71	1.50	10.11	837.15
IPAVFK	Non-toxin	0.16	0.61	1.30	−0.55	0.33	1.00	9.11	673.93
TPEVDK	Non-toxin	−0.36	0.62	−1.50	1.18	0.83	−1.00	4.38	687.82
GLDIQK	Non-toxin	−0.18	0.67	−0.50	0.43	0.83	0.00	6.19	672.87
IIAEK	Non-toxin	0.00	0.66	0.68	0.38	0.60	0.00	6.35	572.77
EALEK	Non-toxin	−0.31	0.62	−1.06	1.34	0.80	−1.00	4.54	588.72

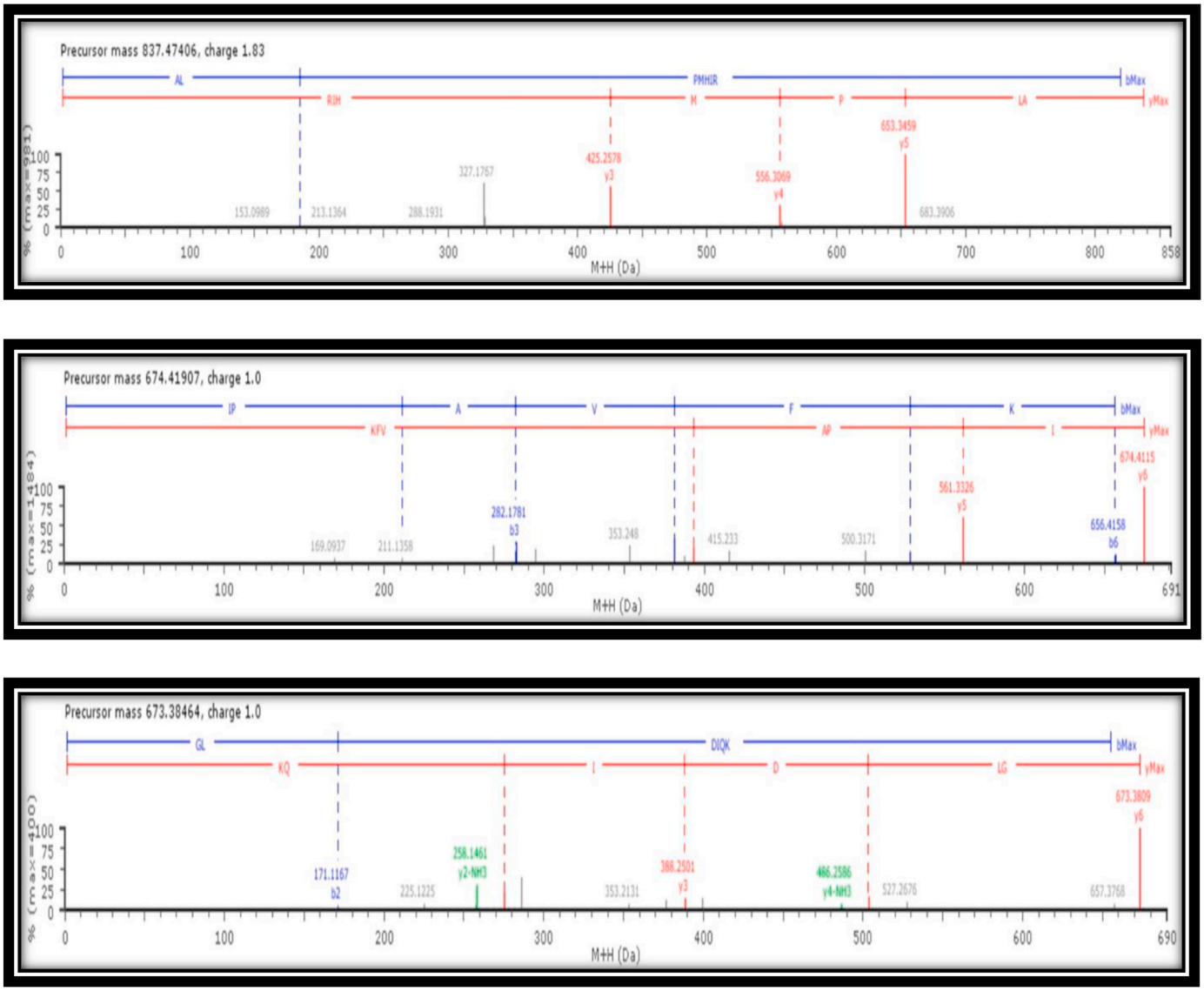

Fig. 2. MS spectrum that corresponds the ionization of ALPMHIR, IPAVFK and GLDIQK peptides.

3.4.3. *GalaxyPepDock*

In GalaxyPepDock server, the interaction of the beta-lactoglobulin derived peptides with SARS-CoV-2 (COVID-19) 6M03 (Apo) and 6LU7 (Holo) enzyme, and lung 1P8J (Furin) were investigated in terms of protein structure similarity (TM-score), interaction similarity score, and estimated accuracy.

The structure similarity scores (TM) for the protein-peptide interaction, which were above 0.98, were shown in Table 7. According to the protein-peptide docking results with 6LU7 and 6M03 proteins, the peptides with an interaction similarity score greater than 50 and an estimated accuracy score greater than 0.70 are IPAVFK, EALEK, ALPMHIR, IIAEK, and GLDIQK peptides. For the 1P8J protein, only ALPMHIR is the peptide that interacts in this score range (Table 7). Fig. 4 shows the docking of SARS-CoV-2 6LU7 protein with the ALPMHIR peptide.

4. Discussion

The global epidemic caused by the new type of human coronavirus, SARS-CoV-2, is a concern for all humanity. In order to find an effective treatment method for COVID-19, all the aspects of SARS- CoV-2 are still being investigated all over the world.

Table 2
Activity of peptides on ACE and DPP-4 inhibitions by BIOPEP.

Sequence	A	B	Activity
ALPMHIR	0.7143	0.0045	ACE inhibitor
	0.8571	0.0002	DPP-4 inhibitor
GLDIQK	0.6667	0.0103	ACE inhibitor
	0.3333	6.37E+05	DPP-4 inhibitor
TPEVDK	0.3333	0.0005	ACE inhibitor
	0.5000	7.032E-5	DPP-4 inhibitor
IPAVFK	1.0000	0.0220	ACE inhibitor
	1.1667	0.0087	DPP-4 inhibitor
EALEK	0.6000	0.0003	ACE inhibitor
	0.4000	0.0003	DPP-4 inhibitor
IIAEK	0.8000	0.0102	ACE inhibitor
	0.8000	6.22E+05	DPP-4 inhibitor

A: The frequency of bioactive fragments occurrence in protein sequence.
B: Potential biological activity of peptide [μM^{-1}].

Table 3
Predicted binding of the beta-lactoglobulin derived peptides on the protein surface from ACE from *Homo sapiens*.

Sequence	Active amino acids	P *	Potentially bound amino acids on the ACE enzyme (PDB: 2YDM)
ALPMHIR	Leu-2, Pro-3, Met-4, His-5, Ile-6, Arg-7	4.47e-05	GLN281, HIS353, ALA354, SER355, HIS383, GLU384, HIS387, GLU411, ASP415, PHE457, LYS511, HIS513, TYR520, TYR523, SER526
IPAVFK	Ile-1, Pro-2, Ala-3, Val-4, Phe-5	0.0001	GLN281, HIS353, ALA354, HIS383, GLU384, HIS387, GLU411, ASP415, PHE457, LYS511, HIS513, TYR520, TYR523, SER526
GLDIQK	Gly-1, Leu-2, Asp-3, Ile-4, Gln-5	0.0014	GLN281, HIS353, ALA354, HIS383, GLU384, HIS387, PHE391, GLU411, PHE457, LYS511, HIS513, TYR520, TYR523, PHE527
IIAEK	Ile-1, Ile-2, Ala-3, Lys-5	0.0009	TRP279, GLN281, HIS353, ALA354, HIS383, HIS387, PHE391, GLU411, PHE457, PHE460, LYS511, HIS513, TYR520, TYR523
TPEVDK	Thr-1, Pro-2, Glu-3, Val-4, Lys-6	0.0009	GLN281, HIS353, HIS383, GLU384, HIS387, HIS410, GLU411, ALA412, ILE413, ASP415, PHE457, LYS511, HIS513, TYR520, TYR523, SER526, PHE527, GLN530
EALEK	Glu-1, Ala-2, Leu-3, Lys-5	0.0091	GLN281, HIS353, ALA354, HIS383, GLU384, HIS387, GLU411, PHE457, LYS511, HIS513, TYR520, TYR523
IPP	Ile-1, Pro-2, Pro-3	9.736e-07	GLN281, HIS353, ALA354, HIS383, GLU384, GLU411, PHE457, LYS511, HIS513, TYR520, TYR523

*: P value presents the statistical significance. ACE inhibitor IPP peptide was used as positive control (Watanabe et al., 2015).

Since peptides have recently been considered as promising agents against various pathologic conditions (Baig et al., 2018), the design of peptide-derived drugs often requires structural characterization of the underlying protein-peptide interactions. The present *in silico* study aims to evaluate whether the beta-lactoglobulin derived peptides could be effective against the main protease enzyme and spike protein of SARS-CoV-2 and host cell furin. Beta-lactoglobulin derived peptides demonstrated various biological effects that include the inhibition of ACE and DPP-4 (Pérez and Calvo, 1995; Pihlanto-Leppälä, 2000; Walzem et al., 2002). However, there is no study related to their potential antiviral effects in the literature. Thus, the present hypothesis-driven peptidomics study focused on the antiviral effects of goat milk beta-lactoglobulin derived peptides. The LC-Q-TOF/MS method was used to determine peptide sequences hidden in goat milk-based beta--lactoglobulin and ALPMHIR, IPAVFK, GLDIQK, IIAEK, EALEK, and TPEVDK were selected which demonstrated inhibitory effects on ACE and DPP-4 activities based on BIOPEP calculations.

Table 4
Predicted binding of the beta-lactoglobulin derived peptides on the protein surface from DPP-4 enzyme from *Homo sapiens*.

Sequence	Active amino acids	P *	Potentially bound amino acids on the DPP-4 enzyme (PDB: 3WQH)
ALPMHIR	Ala-1, Leu-2, Pro-3, Met-4, Ile-6, Arg-7	0.0004	TYR48, VAL546, TYR547, TRP627, GLY628, TRP629, SER630, TYR631, TYR666, GLY741, HIS748, TYR752
IPAVFK	Ile-1, Pro-2, Ala-3, Val-4	0.0123	TYR48, TRP627, GLY628, TRP629, SER630, GLY741, HIS748, TYR752
GLDIQK	Leu-2, Asp-3, Gln-5, Lys-6	0.0370	TYR547, TRP627, GLY628, TRP629, SER630, TYR631, VAL653, TYR666, TYR752
IIAEK	Ile-1, Ile-2, Ala-3, Lys-5	0.0250	GLU206, TYR547, TRP627, GLY628, TRP629, SER630, VAL653, TYR662, TYR666
TPEVDK	Thr-1, Pro-2, Glu-3, Val-4, Lys-6	0.0034	TYR48, VAL546, TRP627, GLY628, TRP629, SER630, HIS748, TYR752
EALEK	Glu-1, Ala-2, Leu-3, Lys-5	0.0143	PHE357, TYR547, SER630, TYR631, TYR634, VAL656, TRP659, TYR662, TYR666, VAL711, HIS740
IPI	Ile-1, Pro-2, Ile-3	0.0025	TYR48, VAL546, TYR547, TRP627, GLY628, TRP629, SER630, TYR631, TYR666, GLY741, HIS748, TYR752

*: P value presents the statistical significance. DPP-4 inhibitor IPI (Diprotin A) peptide was used as positive control (Song et al., 2017).

Table 5
Relative ranking of peptide binding: HPEPDOCK study.

Sequence	Docking score				
	6M03	6LU7	6VSB	6VXX	1P8J
ALPMHIR	−175.882	−172.218	−170.893	−203.151	−179.643
IPAVFK	−163.661	−140.591	−145.968	−163.756	−168.932
GLDIQK	−130.144	−119.002	−115.667	−133.070	−129.011
IIAEK	−131.396	−102.321	−132.848	−130.059	−121.846
TPEVDK	−121.307	−109.671	−108.771	−119.279	−122.394
EALEK	−114.968	−99.104	−105.838	−105.281	−106.875

PDB codes; 6M03: The crystal structure of COVID-19 main protease in apo form.
6LU7: The crystal structure of COVID-19 main protease in complex with an inhibitor N3.
6VSB: Prefusion 2019-nCoV spike glycoprotein with a single receptor-binding domain up.
6VXX: Structure of the SARS-CoV-2 spike glycoprotein (closed state).
1P8J: Crystal structure of the proprotein convertase furin.

The potential therapeutic target would be the disruption of binding of SARS-CoV-2 to host cell protease by beta-lactoglobulin derived peptides. This present *in silico* docking analysis was performed to find a probable ligand-receptor confirmation between the host cell furin, SARS-CoV-2 main protease, and spike protein and beta-lactoglobulin derived peptides by using pepATTRACT, HPEPDOCK, and Galaxy Pep DOCK databases.

In many viruses, proteases play essential roles in viral replication. Therefore, proteases are often used as targets during the investigation of new antiviral agents. When beta-lactoglobulin derived peptides and both holo and apo forms of SARS-CoV-2 main protease were docked, ALPMHIR, IPAVFK, and GLDIQK were found to demonstrate the lowest docking scores. Since the main protease is essential for the maturation of the SARS-CoV-2 and has been examined as a potential target protein, ALPMHIR, IPAVFK, and GLDIQK showed a predicted inhibitory effect on SARS-CoV-2 main protease.

Furthermore, when spike protein of SARS-CoV-2 and beta-lactoglobulin derived peptides were docked, our results have shown that these beta-lactoglobulin derived peptides could bind to the SARS-CoV-2 spike protein (PDB:6VXX) and its receptor binding site (PDB:6VSB). ALPMHIR peptide had the lowest docking score among the other peptides, indicating better interactions with spike proteins of

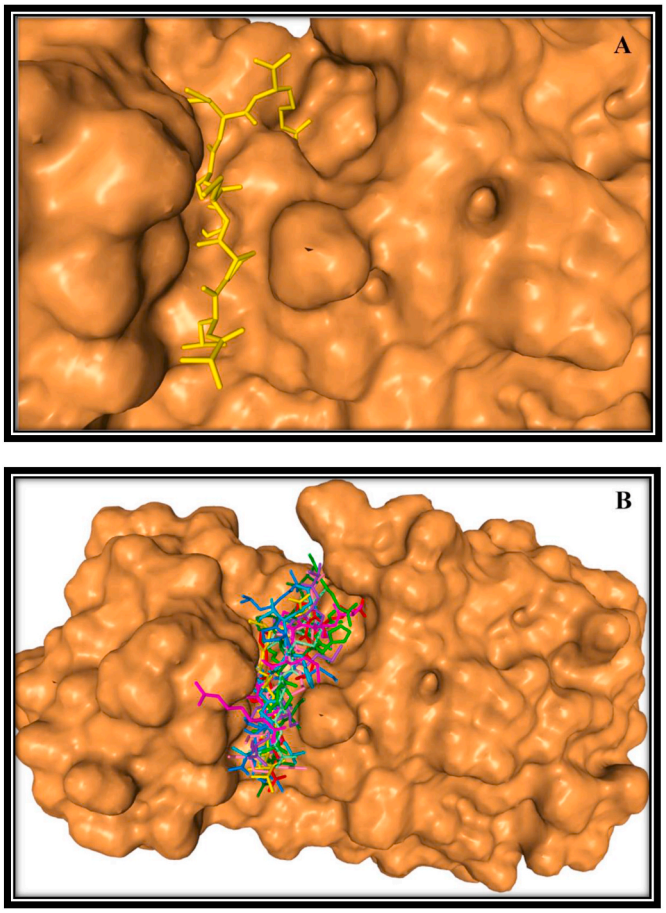

Fig. 3. Molecular docking of SARS-CoV-2 main protease (PDB:6LU7) protein and ALPMHIR peptide **(A):** In the interaction of SARS-CoV-2 main protease (PDB:6LU7) protein with. ALPMHIR peptide **(B):** 10 different interactions of ALPMHIR peptide.

Table 6
Residue, contacts and energy scores obtained from the interaction of peptides with the crystal structure of SARS-CoV-2 parent protease in Apo form (PDB: 6M03) and SARS-CoV-2 main protease in complex with an inhibitor N3(PDB: 6LU7).

Sequence	6M03			6LU7		
	Residue	Contacts	Energy	Residue	Contacts	Energy
ALPMHIR	294	63.34	−13.68	107	36,14	−13.92
IPAVFK	105	35.74	−12.81	110	22.46	−12.96
GLDIQK	126	59.94	−11.37	126	63,74	−11.97
IIAEK	126	29.78	−11.93	294	45,62	−11.34
EALEK	126	29.64	−11.08	126	34,5	−10.65
TPEVDK	4	48,68	−9.75	4	49,2	−9.67

Residue: The number of amino acid that the peptide bound on the protein, **Contacts:** Interface propensity, **PDB codes; 6M03:** The crystal structure of COVID-19 main protease in apo form **6LU7:** The crystal structure of COVID-19 main protease in complex with an inhibitor N3.

SARS-Co-2. Therefore, ALPMHIR might prevent the binding of SARS-CoV-2 to the host cell.

The spike protein of SARS-CoV-2 contains a potential cleavage site for furin. Furin is a proprotein convertase that proteolytically cleaves SARS-CoV-2 spike protein at the S1–S2 boundary by inserting 12 nucleotides to that site (Shang et al., 2020). According to the virtual screening data obtained in this study, beta-lactoglobulin derived these six peptides bound to the furin with low docking scores. Among them, ALPMHIR, IPAVFK, and GLDIQK peptides, which demonstrated the lowest score, should be evaluated as candidate agents in terms of furin inhibition during COVID-19.

Table 7
Protein structure similarity, interaction similarity score and estimated accuracy values after protein peptide dockings in GalaxyPepDock.

Sequence	PDB	Protein structure similarity (TM-score)	Interaction similarity score	Estimated accuracy
ALPMHIR	6LU7	0.989	67.0	0.765
	6M03	0.990	67.0	0.767
	1P8J	0.992	51.0	0.733
IPAVFK	6LU7	0.989	88.0	0.812
	6M03	0.990	88.0	0.814
	1P8J	0.992	43.0	0.715
GLDIQK	6LU7	0.989	64.0	0.759
	6M03	0.990	64.0	0.760
	1P8J	0.992	45.0	0.720
IIAEK	6LU7	0.989	66.0	0.763
	6M03	0.990	66.0	0.765
	1P8J	0.992	45.0	0.720
EALEK	6LU7	0.989	80.0	0.794
	6M03	0.990	80.0	0.796
	1P8J	0.992	43.0	0.715
TPEVDK	6LU7	0.989	48.0	0.723
	6M03	0.990	48.0	0.724
	1P8J	0.992	47.0	0.724

Human coronaviruses have been shown to bind to host cell receptors that include furin, ACE2, and DPP-4 and to use them to invade target cells (Millet and Whittaker, 2015; Bosch et al., 2014). Iacobellis (2020) stated that DPP-4 might be a potential target to prevent multi-organ damage and reduce the risk of acute respiratory complications in patients with type 2 diabetes during COVID-19 infection.

Dilemma on the risk of using ACE inhibitors during the COVID-19 outbreak in patients having the concurrent cardiovascular disease has been discussed due to potential enhancement of the entry of SARS-CoV-2 to host cells via the upregulation of ACE2. However, it is well known that Ang 1–7 produced by ACE2 has beneficial effects in the renin-angiotensin-aldosterone system and could prevent multi-organ damage by counteracting the harmful effects of AII (Li et al., 2020; Warner et al., 2004; South et al., 2020). Similarly, Liu et al. (2020) demonstrated that viral load and lung injury were associated with an increase of serum angiotensin II levels in patients with COVID-19.

5. Conclusion

In conclusion, this *in silico* study used a hypothesis-driven peptidomics strategy for selecting the beta-lactoglobulin derived peptides that inhibit the ACE, DPP-4, and furin enzymes in the host cell and SARS-CoV-2 main protease and that bind to the spike protein of SARS-CoV-2. Among the six peptides examined, ALPMHIR and IPAVFK might be candidates to exert an antiviral activity on SARS-CoV-2. According to the results of this *in silico* analysis, ALMPHIR and IPAVFK peptides were identified as potential candidates to be used in the treatment of SARS-CoV-2 after the future in *vitro* and in *vivo* studies.

Author agreement

An Author Agreement is a statement to certify that all authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

CRediT authorship contribution statement

Bilal Çakır: Conceptualization, Methodology, Formal analysis, Resources. **Betul Okuyan:** Conceptualization, Writing - review & editing. **Göksel Şener:** Conceptualization, Writing - review & editing. **Tugba**

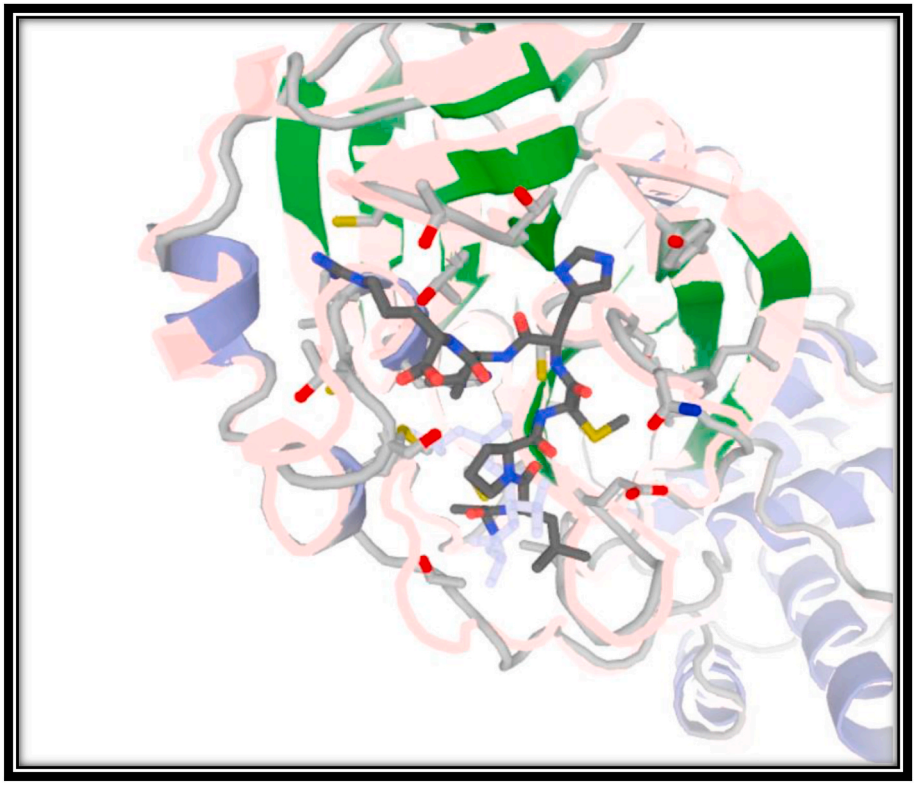

Fig. 4. Docking between ALPMHIR peptide and 6LU7 protein (GalaxyPepDock).

Tunali-Akbay: Conceptualization, Methodology, Project administration, Resources.

Declaration of competing interest

The authors declare "No conflict of interest".

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