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Potential anticarcinogenic effect of goat milk-derived bioactive peptides on HCT-116 human colorectal carcinoma cell line

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ABSTRACT

Novel food-derived anti cancerogenic bioactive peptides were characterized by goat milk pepsin hydrolysate. Pepsin treated casein fraction of goat milk caused an apoptotic cell death on the HCT116 cell lines. These bioactive peptides are encrypted in the protein structure in the inactive form and can become active during gastrointestinal digestion in the body. In this study, the possible therapeutic effect of goat milk-based bioactive peptides on human colorectal cancer cell lines was investigated. Goat milk-derived bioactive peptides were extracted from the casein and whey protein fractions using trypsin, pepsin, and papain enzymes. The bioactive peptides were characterized by the liquid chromatography quadrupole time of flight mass spectrometry. Both enzyme-treated casein and whey fractions were incubated with the HCT116 cell lines, and then the cell cytotoxicity was evaluated using MTT assay. The type of cell death was analyzed by flow cytometry using Annexin V and propidium iodide.

Among all applications, the pepsin-treated casein fraction was the highest potential peptides that cause 80.92% apoptotic cell death. In conclusion, pepsin treated casein fraction exhibited antiproliferative activity against HCT116 cells. The bioactive peptides of this fraction can be considered as a potential source for the development of new anti cancerogenic agents.

1. Introduction

Bioactive peptides are specific protein fragments that affect the physiological and metabolic functions of the body and have beneficial effects on human health. They are not active in the main protein sequence [1], but are activated by enzymatic hydrolysis via digestive enzymes, fermentation of milk through proteolytic starter cultures, and also by the enzymes of proteolytic microorganisms [2]. The source of bioactive peptides is mostly nutrients that are consumed in daily life. Milk proteins are known as an important source for bioactive peptides and can be obtained by the enzymatic hydrolysis of casein and whey protein fractions [3–7]. Milk-based bioactive peptides have anti-oxidative, antihypertensive, antithrombotic, antimicrobial, immunomodulator, and anti-obesity effects [8–10]. Goat milk has some specific properties compared to other milk types. These properties are related to its buffering capacity [11,12] and its easy digestion due to its small-sized fat globules and short-chain fatty acids. Goat milk also reduces the cholesterol levels in human blood by improving cholesterol mobilization and controlling its storage [13,14]. Besides, serum protein hydrolysates of goat milk have been reported to have ACE inhibitory activity, particularly β-lactoglobulin [15]. Park [11] has revealed the antimicrobial properties of goat milk casein peptides. Lopez-Exposito and Recio [16] showed the antibacterial properties of pepsin treated sheep milk, and Park et al. [12] showed the presence of these fragments in the pepsin treated goat milk.

Colorectal cancer is a malignant disease due to its high incidence and mortality. It is an uncontrollable disease that develops slowly, starting with polyps on the epithelial lining of the colon or rectum [17]. The treatment of colorectal cancer involves surgical removal of the tumor followed by adjuvant chemotherapy. Targeted drugs are also combined with chemotherapy. Many natural products have been described repeatedly because of their potential use for the treatment of cancer; preventing the discovery of new compounds [18]. Therefore, the discovery of new structural compounds remains a major challenge. Food-based bioactive peptides have the potential to be a new structural compound. Among the acquiring methods, the enzymatic hydrolysis method is the most suitable and widely used method for preparing bioactive peptides as the have high efficiency and stability, and ease of

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Table 1
The optimal conditions for the proteases.

Enzyme	pH	Temprature	Solutions and buffers
Pepsin, 0.2 mg/mL	2	25 °C	0.05 N HCl
Trypsin, 0.2 mg/mL	8	37 °C	0.02 M Tris.HCl
Papain, 0.2 mg/mL	7	37 °C	0.02 M Sodium phosphate

analyses compared to microbial fermentation [19]. Therefore, this study aims to investigate the effects of bioactive peptides that were obtained from the pepsin, trypsin, and papain treatment of goat milk on the HCT 116 colorectal cancer cell lines.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich Corp (St. Louis, MO, USA) and used without further purification. Materials used for proteomic analysis were obtained from Expedeon (Expedeon Ltd, Cambridge, UK) and Thermo Fisher Scientific (Thermo Fisher Scientific, Glasgow, UK).

2.2. Goat milk

Goat milk was obtained from the Saanen-Maltiz crossbreed goats that were grown in a farm located in the Thrace region of Turkey. Goat milk was stored at +4 °C after milking in the morning and was brought to the laboratory within 1–1.5 h without cold chain deterioration.

2.3. Nutritional value

Goat milk nutritional value was tested by the determination of protein [20], fat [21], energy [22], moisture [23], dry matter [23] and ash [24] levels.

2.4. Protein extraction

The milk sample is diluted 1:15 with distilled water. The pH is adjusted to 9.5 using 1 N NaOH solution to allow the proteins to dissolve. It is then stirred for 1 h at room temperature with a magnetic stirrer and centrifuged at 5000×*g* for 30 min. The supernatant is separated and

pH was adjusted to 4.5 with 1 N HCl solution. The precipitate portion includes casein, and the supernatant portion contains serum proteins. The casein and serum proteins were then lyophilized and stored at 4 °C [25].

2.5. Digestion of goat milk protein hydrolysates with proteases

Three kinds of proteases (pepsin, trypsin, and papain) were used for the digestion of goat milk protein hydrolysates; digestion conditions (solution, pH, and temperature) shown in Table 1. Goat milk protein hydrolysates were dissolved in (10 mg/mL) and digested with pepsin at 25 °C for 24 h or either trypsin or papain at 37 °C (ratio of protein substrate to enzyme, 100:5, wt/wt). After digestion, each sample was heated at 98 °C for 10 min to inactivate the protease [26].

2.6. Analysis of peptide sequences by LC-Q-TOF-MS

For the extraction of proteins, the cell pellet was mixed with Universal Protein Extraction (UPX) Kit (Expedeon-44101) and protease inhibitor cocktail (Thermo Sci.-87785). Peptide production was performed using FASP Protein Digestion Kit (Expedeon-44250) and trypsin enzyme (Pierce-90057). The final concentration of the sample was 200 mg/L. Protein identification experiments were performed using LC-Q-TOF-MS (Waters, Xevo G2-XS, USA) system at Acıbadem University, LABMED, Turkey. UniProt protein database and Progenesis QIP software were used for protein identification.

2.7. Incubation of HCT 116 cells with either pepsin, trypsin or papain treated goat milk whey and casein fractions

Cell culture and cellular viability determinations were carried out at GEMHAM, Marmara University, İstanbul, Turkey. HCT116 colon carcinoma cells (Sigma-Aldrich, CLL1222, USA) were grown on McCoy's medium (Sigma-Aldrich, M9309, USA). When the pre-determined cell concentration range was reached, the number of viable cells was determined using trypan blue and the cells were inoculated in Petri dishes (60 mm) at a cell concentration of 3 × 106. Cells were incubated at 37 °C under 5% CO_2 for 24 h to facilitate the adhesion. On the following day, each digested goat milk protein fractions (casein and whey proteins) were added to the medium separately. Upon the completion of a 48 h incubation period (5% CO_2), the effect of each treatment on cellular viability was determined with MTT assay.

2.8. Cell viability (MTT assay)

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay is a colorimetric assay that is widely utilized in the evaluation of cytotoxic properties of bioactive compounds [27]. In this essay, the number of viable cells after an anti-cancerogenic treatment is determined based on the formation of purple formazan crystals by the enzymes in viable cells. Immediately after 48 h incubation with the treatment samples, media were removed from the Petri dishes, and fresh media (3 mL) that contained 100 μl MTT was added. The cells were kept incubated with MTT for another 2–4 h (37 °C, 5% CO_2). Once again, the media were removed, and a 1 mL solubilizing solution was added to the Petri dishes (5 min incubation at room temperature). Immediately afterward, absorbance values (590 nm) were compared for treatments and a control sample to determine the extent of viability using a UV/Vis Multiwell Reader (96 well-plates and 50 μl/well).

% Cellular viability was based on the absorbance values:

% Viability = [(Sample absorbance – blank) / Control absorbance - Blank)] x 100

2.9. Evaluation of apoptosis and necrosis by flow cytometry

The apoptotic and necrotic potential of the goat milk protein fractions (casein and whey proteins) was evaluated using flow cytometry (BD, FACS Calibur 4CS, USA). HCT116 cells (1 × 10^6 cells) were cultivated in a T25 culture flask in triplicate experiments along with the control flasks containing the unstained, only Annexin V stained or only propidium iodide (PI) stained cells. The total volume was 500 μl, which consisted of 400 μl of cells and 100 μl of incubation buffer which was free of stains or contained either Annexin V or PI (2 μl, at a concentration of 1 mg.ml-1). After 24 h incubation under appropriate conditions (37 °C, 5% CO_2), goat milk protein fractions (casein and whey proteins) were administered at varying concentrations. Following a 24 h incubation with the treatments, the adherent cells were trypsinized in each T25 flask. The collected cells were washed with phosphate-buffered saline (PBS) twice and centrifuged (670×*g*, 5 min) immediately afterward. The precipitated cells were resuspended once again in PBS and prepared for the flow cytometric analysis. The tubes contained 400 μL cellular suspension, 10 μL incubation buffer, 2 μL Annexin-V, and 2 μL PI. The tubes were analyzed using a flow cytometry instrument (BD FACS Calibur 4CS Flow Cytometer, BD Biosciences, San Jose, CA, USA). Cells that were negative for both PI and Annexin-V negative were considered healthy. PI negative and Annexin positive cells were considered apoptotic, whereas

Table 2
Analysis of goat milk.

Analysis	Mean ± SD
pH	6.70 ± 0.06
Moisture, % (m/m)	87.20 ± 0.34
Dry matter, % (m/m)	12.80 ± 0.34
Ash, % (m/m)	1.01 ± 0.06
Protein, % (m/m)	3.84 ± 0.06
Fat, % (m/m)	4.00 ± 0.32
Energy, kcal/100 g	66.84 ± 1.20

SD: Standard deviation, n = 10.

cells that were stained with both stains were considered late apoptotic or necrotic since these staining methods do not differentiate the late apoptotic or necrotic cells [28,29].

2.10. Peptide characterization

Potential biological activities of the peptides were obtained using the BioPep tool (Table 4, http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, accessed September 10, 2020). The presence of peptides that are found in goat milk protein was confirmed using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed September 12, 2020).

2.11. In silico analysis of peptide sequences

The obtained peptide sequences were selected using the Peptide Ranker webserver [30] accessed at http://bioware.ucd.ie/. PeptideRanker is a server that predicts the probability that each of the peptides will be bioactive or not. Based on the score of above 0.5, peptides were listed as potential bioactive peptides. Any peptide predicted over a 0.5

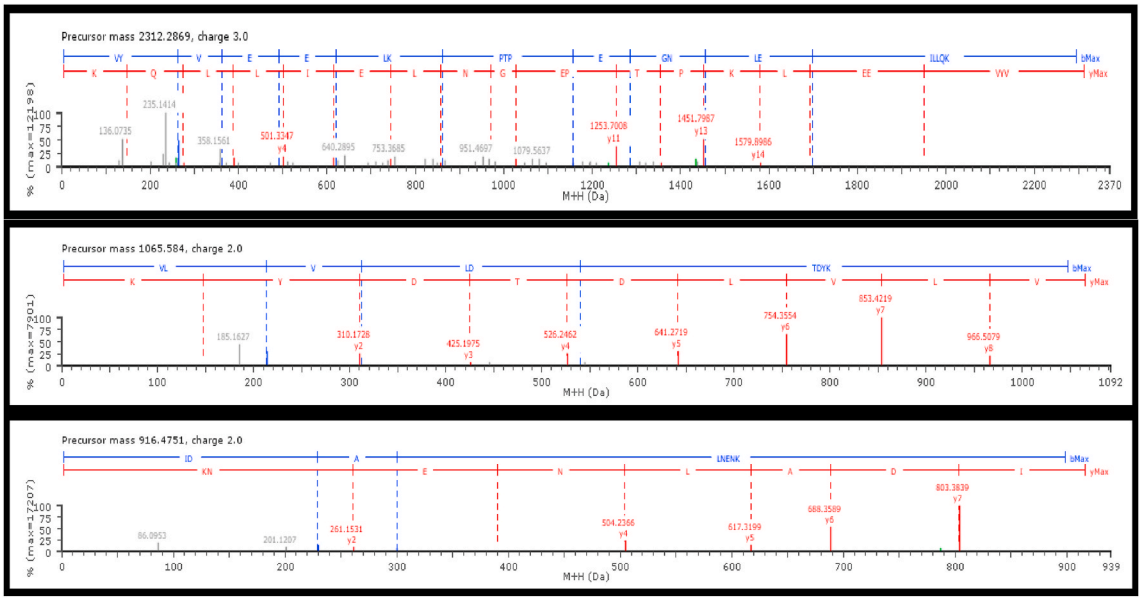

Fig. 1. Whey protein peptide sequence study.

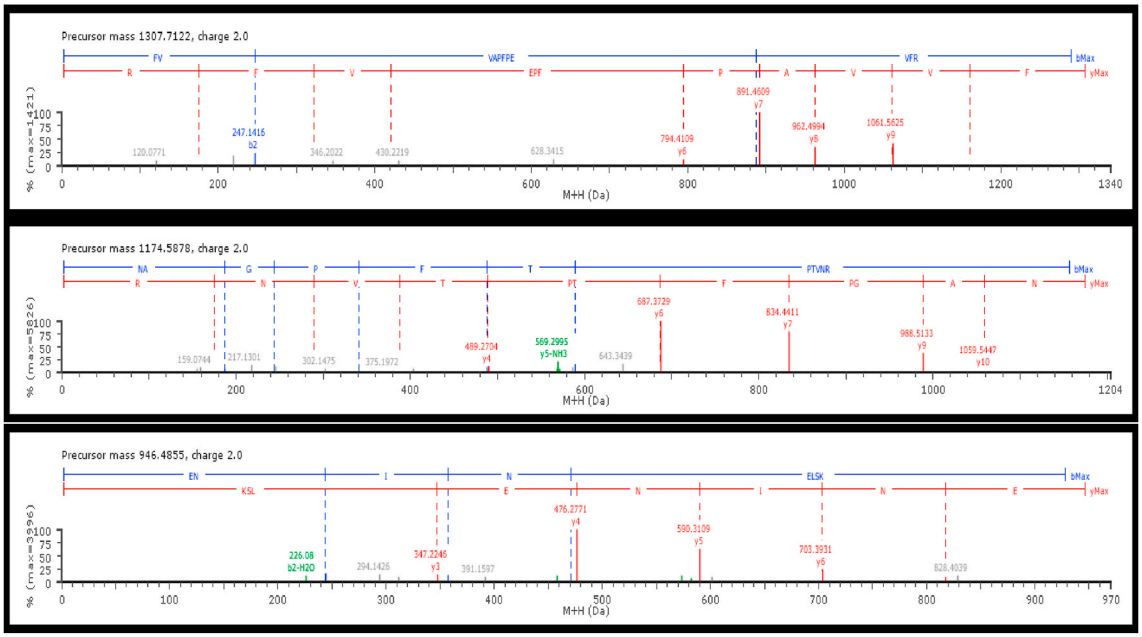

Fig. 2. Casein protein peptide sequence study.

Table 3
Sequences of potential biologically active goat milk peptides with their score generated from PeptideRanker.

No	Score	Sequence
1	0.906686	FAWPQYLK
2	0.824852	QYKIPDWFLNR
3	0.753432	DYRWIAL
4	0.752583	QFTPEKFFGIFNK
5	0.748999	GIIFNNNPALWK
6	0.737646	FVVAPFPEVFR
7	0.724140	ARHPHPHL
8	0.671080	QIMSSPWGEMYNIF
9	0.626675	ALPMHIR
10	0.622517	CDELGIMIWQDF
11	0.591682	QYPYQGPIVL
12	0.567136	SRYPSYG
13	0.557860	SSGLGNVPRPYQL
14	0.513282	LVMFQRR

threshold is labeled as bioactive in PeptideRanker, and the user may decide to choose a higher threshold to reduce the number of false positives.

2.12. Statistical analysis

SPSS 25.0 software was utilized to ensure statistical validity. For cell culture assay, six replicate experiments were carried out and were evaluated using ANOVA ($p < 0.05$).

3. Results

3.1. Nutritional value of goat milk

The nutritional parameters of goat milk were presented in Table 2.

Table 4
Peptides obtained from the pepsin treated casein fraction of goat milk.

RT (min)	Charge	*m/z*	Meas. Mass	Sequence	Accession	Description	Potential biological activities (BIOPEP)
20.8518	3	439.8986	1316.67	YPWKPTSKSEL	Q95327	Beta-mannosidase	ACE inhibitory activity, antioxidant activity, dipeptidyl peptidase IV inhibitory activity, vasoactive substance release stimulating activity, alpha-glucosidase inhibitory activity
21.8014	2	764.3337	1526.65	CDELGIMIWQDF[a]	Q95327	Beta-mannosidase	ACE inhibitory activity, antioxidant activity, dipeptidyl peptidase IV inhibitory activity
20.1025	4	397.2278	1584.88	EKSAKVYFRTVEL	Q95327	Beta-mannosidase	ACE inhibitory activity, antioxidant activity, dipeptidyl peptidase III inhibitory activity, dipeptidyl peptidase IV inhibitory activity
21.4296	2	468.7610	935.51	DYRWIAL[a]	Q95327	Beta-mannosidase	ACE inhibitory activity, antioxidant activity, ion flow regulating activity, dipeptidyl peptidase IV inhibitory activity and neuropeptide activity
19.6792	2	593.3111	1184.61	TVRVHTWSSL	Q95327	Beta-mannosidase	ACE inhibitory activity, antioxidant activity, dipeptidyl peptidase III inhibitory activity, dipeptidyl peptidase IV inhibitory activity
30.5067	2	589.3004	1176.59	QYPYQGPIVL[a]	P33049	Alpha-S2-casein	ACE inhibitory activity, antioxidant activity, dipeptidyl peptidase IV inhibitory activity, alpha-glucosidase inhibitor activity, antithrombotic activity, antiamnestic activity, Glucose uptake stimulating activity, stomach mucosal membrane regulating activity.
23.7690	2	640.3194	1278.62	TEEEKNRLNF	P33049	Alpha-S2-casein	ACE inhibitory activity, vasoactive substance release stimulating activity, renin inhibitory, dipeptidyl peptidase IV inhibitory activity.
22.3963	3	633.3536	1897.04	KQEKNMAIHPRKEKL	P33049	Alpha-S2-casein	ACE inhibitory activity, antioxidant activity and dipeptidyl peptidase III and dipeptidyl peptidase IV inhibitory activity.
22.1217	3	568.2682	1701.78	QIMSSPWGEMYNIF[a]	O18809	Cytochrome P450	ACE inhibitory activity, antioxidant activity and dipeptidyl peptidase III and dipeptidyl peptidase IV inhibitory activity.
22.4478	2	723.8676	1445.72	SSGLGNVPRPYQL[a]	O18809	Cytochrome P450	ACE inhibitory activity, antioxidant activity and dipeptidyl peptidase III and dipeptidyl peptidase IV inhibitory activity.

[a] Potential bioactive peptide sequence that has been determined by the Peptide Ranker ($p > 0.5$).

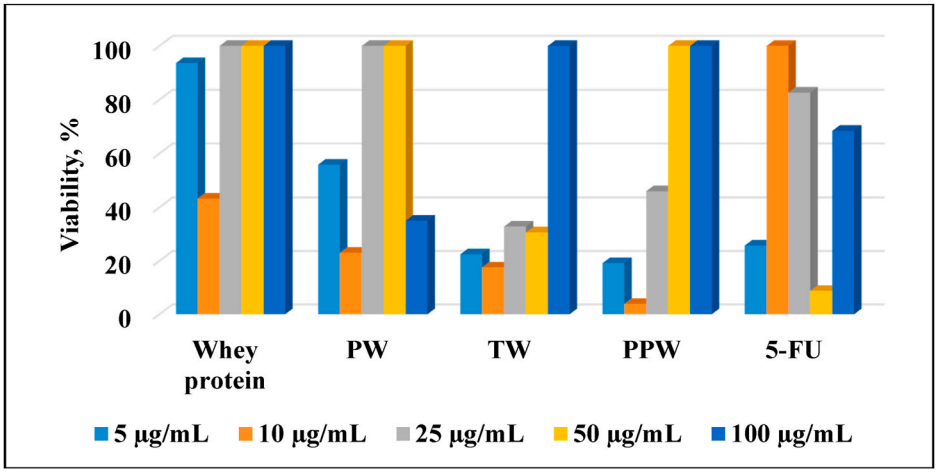

Fig. 3. Cell viability analysis of HCT116 colon cancer cells after 48 h incubation with enzymatic digestion products of goat milk whey proteins goat milk whey protein fractions prepared at different concentrations. **(PW:** Pepsin-treated whey proteins fraction, **TW:** Trypsin-treated whey proteins fraction, **PPW:** Papain-treated whey proteins fraction, **5-FU:** 5-Fluorouracil).

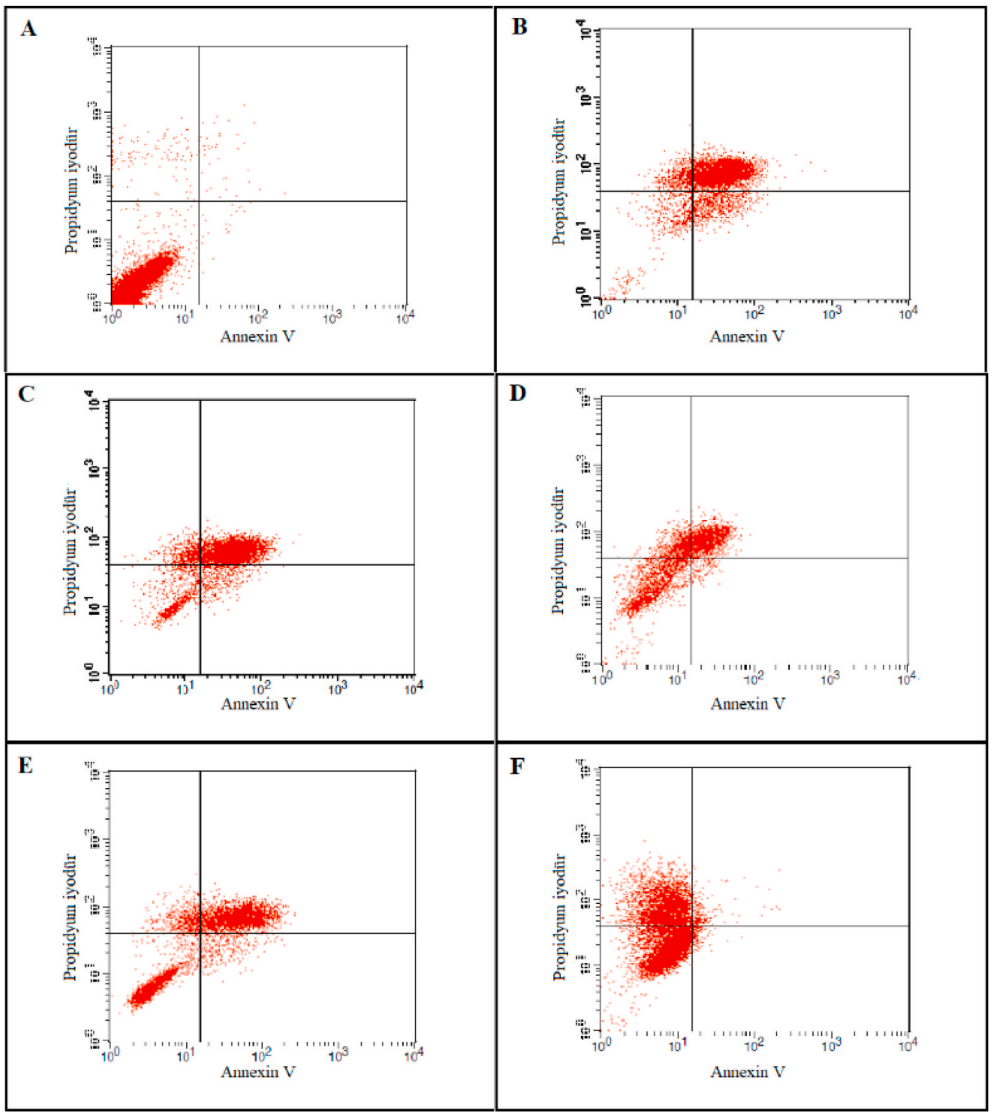

Fig. 4. Analysis of apoptosis by flow cytometry in HCT 116 cells that were treated with whey proteins and whey peptide fractions. (**A:** Control Cells, **B:** Whey Proteins, **C:** Pepsin-treated whey proteins fraction, **D:** Trypsin-treated whey proteins fraction, **E:** Papain-treated whey proteins fraction, **F:** 5-Fluorouracil).

3.2. Peptides produced by enzymatic digestion of casein and whey proteins

A total of 93 different peptide sequences were found, among which 51 peptides were from casein proteins and 42 peptides from whey proteins. Studies on the sequence analysis of the obtained peptides were expressed in spectrograms (Fig. 1 and Fig. 2). A total of 93 peptides obtained from the above section were subsequently screened and selected using the Peptide Ranker webserver [30] accessed at http://bioware.ucd.ie/. Based on the score of above 0.50, peptides were listed as potential bioactive peptides (Table 3). ARHPHPHL peptide, ALPMHIR peptide, and SRYPSYG peptide have been found to have some bioactivities. The peptide sequence ARHPHPHL has been found to have potential antioxidant and ACE inhibitory effect. ALPMHIR (betalactokinin) has been found to have an ACE inhibitory effect with an exact match. SRYPSYG (casoxin) has been found to have a potential opioid antagonist.

3.3. Selective cytotoxicity and cell death induced by whey protein fractions against HCT116 colon cancer cells

Fig. 3 shows the effects of 5, 10, 25, 50, and 100 μg/mL pepsin, trypsin, or papain treated whey protein fractions on HCT 116 cell viability. The highest cell death rate was obtained with the 10 μg/mL whey protein fraction treated with pepsin, trypsin, and papain compared to the other doses.

3.4. Analysis of apoptosis in HCT 116 cells that were incubated with whey protein and whey peptide fractions

10 μg/mL whey protein and whey peptide fractions caused the highest HCT 116 cell death in MTT analysis. Flow cytometric analysis was carried out with 10 μg/mL concentration. 5- fluorouracil (10 μg/mL) was used as a positive control. The percentage of Annexin V positive cells was higher in whole whey protein treated HCT cells, and whey peptide fractions treated HCT116 cells when compared to the positive control (Fig. 4). Flow cytometric analysis also revealed that whey protein fractions induced a dose-dependent increase in apoptotic cell death. After the HCT 116 cells were treated with whole whey proteins, pepsin treated whey (PW) fraction, trypsin treated whey (TW) fraction, and papain treated whey (PPW) fraction, the percentage of HCT 116 cell viability significantly decreased. The average proportion of Annexin V-stained positive cells (apoptotic cells) increased from 0.53% in control to 82.47% in the whole whey protein treatment, to 77.03% in the PW

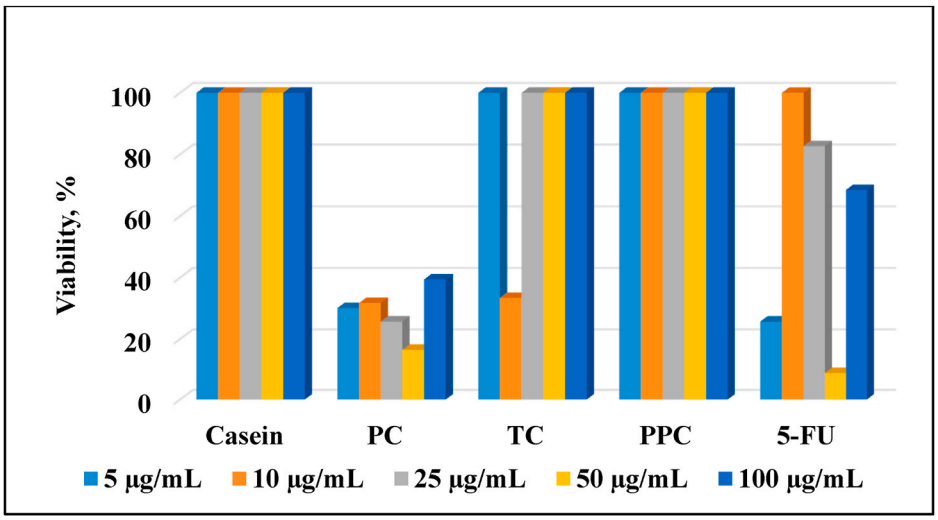

Fig. 5. Cell viability analysis of HCT 116 colon cancer cells after 48 h incubation with enzymatic digestion products of goat milk casein proteins and goat milk casein protein fractions prepared at different concentrations. (**PC:** Pepsin-treated casein fraction**, TC:** Trypsin-treated casein fraction**, PPC:** Papain-treated casein fraction**, 5-FU:**5-Fluorouracil).

treatment, to 40.52% in the TW treatment, and 57.71% in the PPW treatment. Based on these results, 10 µg/mL PW fraction was the fraction that caused the highest apoptotic HCT 116 cell death (Fig. 4).

3.5. Selective cytotoxicity and cell death induced by casein fractions against HCT 116 colon cancer cell

The cytotoxicity of casein fractions against HCT 116 cell lines was evaluated by using MTT assay. Fig. 4 shows the effects of 5, 10, 25, 50, and 100 µg/mL pepsin, trypsin, or papain treated casein protein fractions.

The viability percentage of HCT 116 cells was the lowest when incubated with all the concentrations of PC fraction. Table 4 shows the bioactive peptides of PC fraction and their features. 10 µg/mL TC fraction also caused a high HCT 116 cell death (Fig. 5).

3.6. Analysis of apoptosis by flow cytometry in HCT 116 cells that were incubated with casein protein and casein peptide fractions

After the treatment of whole casein protein, PC fraction, TC fraction, and PPC fraction with HCT 116 cells, the percentage of HCT 116 cell viability significantly decreased (Fig. 6) with all treatments. The average proportion of Annexin V-stained positive cells (apoptotic cells) increased from 0.53% to 56.64% in the whole casein protein treatment, to 80.92% in the PC treatment, to 31.91% in the TC treatment, and 68.16% in the PPC treatment.

According to the data obtained in the MTT test, flow cytometric analysis was performed with 50 µg/mL whole casein protein and casein peptide fractions. 5-Fluorouracil (50 µg/mL) was used as a positive control.

4. Discussion

Milk is an important source for foodborne bioactive peptides and has a potential for the development of functional products due to its therapeutic effects on human health. In this study, the potential anti-carcinogenic effect of goat milk-derived bioactive peptides has been demonstrated by investigating their effects on HCT 116 colon cancer cells. In the first stage of this study, casein and whey fractions of goat milk were digested separately either with pepsin, trypsin, or papain to obtain goat milk peptide fractions. The released peptides were not related to the abundance of the parent proteins but the preferences of the proteases. Shu et al. [31] stated that the hydrolyzates obtained from goat's milk have an inhibiting effect on the angiotensin-converting enzyme. They also reported that this inhibiting effect is higher than that of the hydrolyzates obtained from cow's milk. Baum et al. [32] identified 248 endogenous peptides and 18 putative phosphopeptides after the pre-fractionation of milk peptides in raw cow's milk. Dallas et al. [33] also identified more than 245 endogenous peptides in the serum fraction of bovine colostrum. Two subsequent studies focused on the investigation of endogenous peptides in donkey milk samples [34, 35]. Schuchardt and Sickmann [36] suggested using the mass spectrometric techniques for the identification of protein structures as this technique guarantees further improvements in protein identification performance. In this study, protein identification was performed by LC-MS/MS analysis for the peptidomics research in a biochemical context. Many bioactive peptides were detected from the enzyme-treated whey and casein fractions.

13 peptides that have molecular weights between 582,31 and 1681,83 Da containing 5 to 15 amino acids were detected from the pepsin treated goat milk whey fraction. 5 peptides that contain 7-17 amino acids with molecular weights between 816,44 and 1962,03 Da were obtained from the papain treated goat milk whey fraction. 39 peptides that contain 7-23 amino acids with molecular weights between 687,36 and 2332,08 Da were obtained from the trypsin treated goat milk whey fraction.

10 peptides with molecular weights between 935,51 and 1897,04 Da containing 7–15 amino acids were detected from the pepsin treated casein fraction. 23 peptides with molecular weights between 828,38 and 3188,56 Da were obtained from the papain treated casein fraction, 21 peptides with molecular weights between 826,45 and 2311,30 Da containing 7–27 amino acids were detected from the trypsin treated goat milk casein fraction.

Following the characterization of casein and whey peptide fractions, these fractions were incubated separately with HCT 116 colon cancer cell lines for 48 h.

Treatment of these peptides with HCT-116 cells, the highest HCT 116 cell death rate was obtained with pepsin treated goat milk casein fraction. Sharma and Katz [37] have been reported the inhibiting effect of bioactive peptides on the cancer cells' proliferation [37]. However, the effects of milk and dairy products on colorectal cancer development were in dilemma. Aune et al. [38] have revealed in their meta-analysis that, high intakes of milk and total dairy products were associated with a statistically significant reduction in colorectal cancer risk as compared

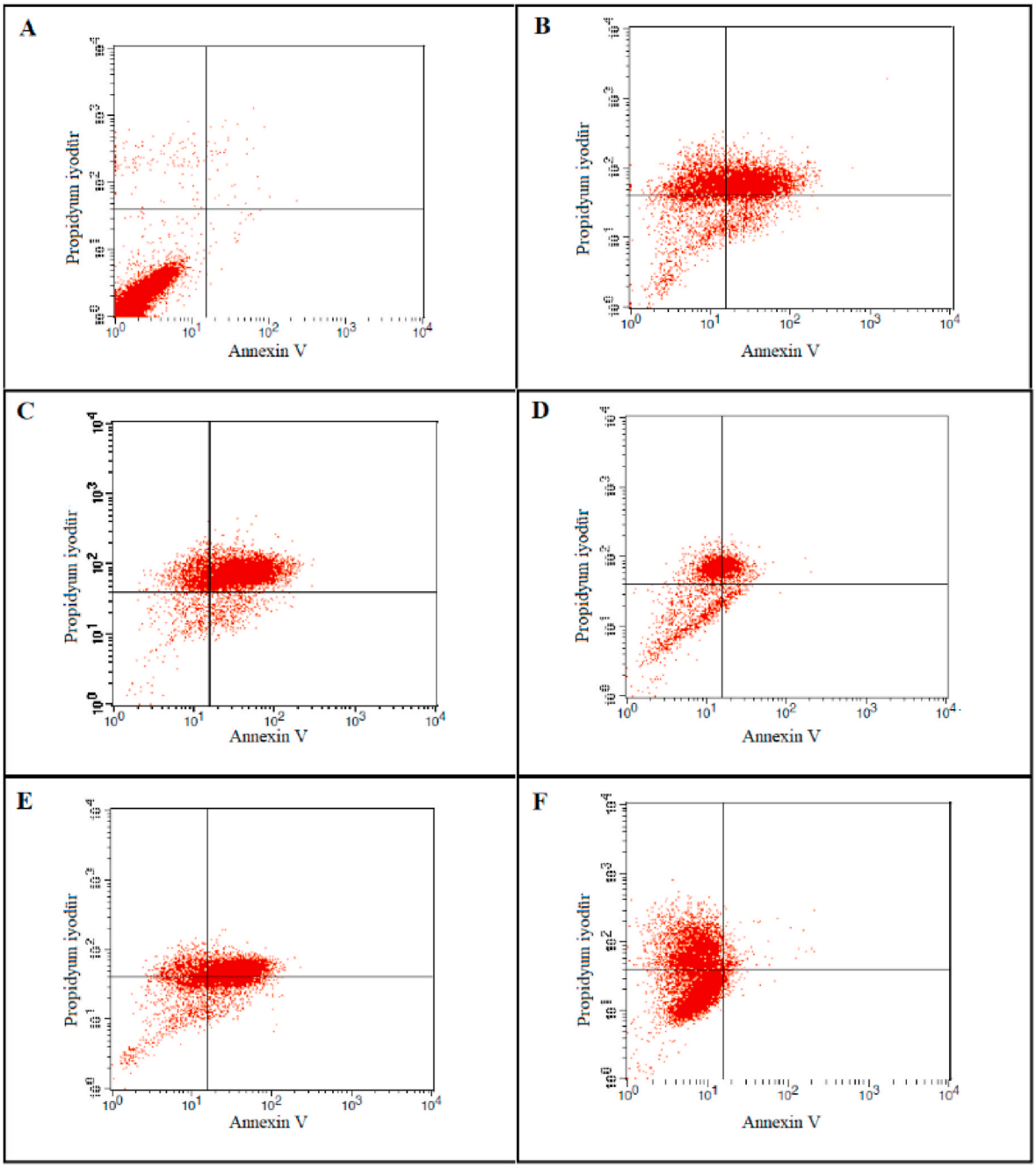

Fig. 6. Analysis of apoptosis by flow cytometry in HCT 116 cells that were treated with casein proteins and casein peptide fractions (**A:** Control Cells, **B:** Casein Proteins, **C:** Pepsin-treated casein proteins fraction, **D:** Trypsin-treated casein proteins fraction, **E:** Papain-treated casein proteins fraction, **F:** 5-Fluorouracil).

with low intake.

Norat and Riboli [39] also attributed the possible protective effect of dairy products to the calcium content and vitamin D, conjugated linoleic acid, sphingolipids, butyric acid, and fermentation products of the body. Cho et al. [40] mentioned the importance of milk consumption and milk calcium that is associated with a lower risk of colorectal cancer.

The anticancer effects of milk bioactive peptides have been reported on different cancer cells. Roy et al. [41] reported that bovine skim milk digested with *Saccharomyces cerevisiae* inhibits the proliferation of a human leukemia cell line. Meisel and FitzGerald stated the anticancer activity of casein phosphopeptides [42]. Milk proteins lactoferrin and lactoferricin have apoptotic, anti-angiogenetic, anti-metastatic, and necrotic effects [43,44].

According to the results of this study, it has been determined that bioactive peptides that were obtained from the pepsin treated casein fraction caused the highest apoptotic cell death rate on the HCT 116 cell line. Although there is apoptotic death in HCT 116 cells treated with trypsin and papain in this study, it would be more directive to discuss and focus on pepsin digestion, as the highest apoptotic cell death in HCT 116 cells treated with pepsin.

The application of bioinformatics technology in foodborne bioactive peptides is currently being studied extensively. The use of bioinformatics can decrease the tests numbers that must be performed to determine how bioactive peptide structure relates to its activity [45]. Compared with the control, 50 μg/mL pepsin treated casein fraction also significantly increased the cell death and the rate of apoptosis. In the pepsin treated casein fraction, 10 peptides were detected. Five of them that are likely to be bioactive peptides were determined using the

PeptideRanker server. BIOPEP server was also used to find the potential biological activity of these 5 bioactive peptides. CDELGIMIWQDF sequence has an ACE inhibitor, antioxidant, and dipeptidyl peptidase IV inhibitory activity. DYRWIAL sequence has an ACE inhibitor, antioxidant, ion flow regulating, dipeptidyl peptidase IV inhibitor, and neuropeptide activity. QYPYQGPIVL sequence has an ACE, dipeptidyl peptidase IV and alpha-glucosidase inhibitor, an anti-oxidant, an anti-thrombotic, glucose uptake stimulating, and stomach mucosal membrane regulating activity. QIMSSPWGEMYNIF and SSGLGNVPRPYQL sequences have been found to have ACE, dipeptidyl peptidase III and dipeptidyl peptidase IV inhibitor and antioxidant activity. The anti-carcinogenic activity that was obtained from the pepsin digested goat milk casein fraction can be attributed to these 5 peptides. The other 5 peptides from the pepsin treated casein fraction that did not exceed the threshold. They may also have the potential to have an anti-carcinogenic activity as there was no study related to their anti-carcinogenic effects in the literature.

After incubating HCT 116 cells with pepsin-treated goat milk casein fraction, protein extraction was performed in the death HCT 116 cells, and peptidomics content was determined and characterized using the LC-Q-TOF-MS system. When matching at least 3 amino acids of the resulting peptides with the proteins in the database, 1498 matches were detected. After the classification of the matched peptides to their relevant proteins, it has been determined that the up-regulated or down-regulated proteins and enzymes were associated with energy metabolism, apoptosis, oxidative stress, and signal transduction mechanisms of HCT 116 cells. Most of these enzymes were related to the energy metabolism of HCT 116 cells, especially related to the glucose usage of the cells and the ATP synthesis.

5. Conclusion

In this study, the anti-apoptotic effect of goat milk-derived bioactive peptides on HCT 116 colon cancer cell lines has been investigated as a novel finding. The results demonstrated that bioactive peptides of the pepsin treated goat milk casein fraction may potentially serve as an alternative food-derived anticancer peptide that could be utilized for the treatment of colon cancer. In light of these results, it should be considered that milk proteins are not only necessary for nutrition but also have the potential for the management of cancer.

Credit author statement

Bilal Cakir: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing- Reviewing and Editing. **Tugba Tunali-Akbay**: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing- Reviewing and Editing.

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