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Original article

Releasing angiotensin I-converting enzyme inhibitory (ACE-I) peptides by *Yamadazyma* spp. in non-fat milk

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Summary The objectives of this study were to analyse the impact of both temperature and different isolates on microbial protease production ability and to examine the hypertensive effect of bioactive peptides (BAPs) that inhibit the angiotensin I-converting enzyme released by fermentation of non-fat milk. First, the ability of several *Yamadazyma* spp. isolates, which were previously isolated and identified from several sources, was screened in terms of the production of proteolytic enzymes at 15 and 25 °C. The effect of temperature on proteolysis activity of selected isolates was considerable, and incubation at 25 °C was favourable for protease production. Besides, combinations of three different isolates showed remarkable proteolytic activity during the incubation periods of 24, 48 and 72 h. The highest angiotensin I-converting enzyme inhibitory (ACE-I) activity was detected for the combination *Yamadazyma* spp. BO10 and B514-1, with an inhibition of 92.5%. The partial purification of peptides by ultrafiltration (cut-off 10 kDa) has no crucial impact on ACE-I activity, and further purification might improve their effectiveness. The implications of fermentation with co-culture may have symbiotic potential to increase the antihypertensive effect of the product. Therefore, these results suggest that *Yamadazyma* spp. may be used to produce functional products or bioactive peptides presenting health-promoting attributes.

Keywords Angiotensin I-converting enzyme, antihypertensive activity, bioactive peptides, fermented milk, *Yamadazyma*.

Introduction

Health concerns and the preference for more natural ingredients, which also impact decreasing the risk of diseases, are major trends followed by consumers recently (Asioli *et al*., 2017). Therefore, studies have focused on natural active components and their relevance, if any, in regulating metabolic pathways (Gianfranceschi *et al*., 2018). Among these components, bioactive peptides (BAPs), which are short-chain protein fragments composed of two to twenty amino acids, have gained attention because of their health-promoting activities (Chalamaiah *et al*., 2019). The biological activity characteristics of BAPs change depending on the type, sequence and molecular weight of amino acids (Görgüç *et al*., 2020). Apart from antimicrobial (Freitas *et al*., 2019), antioxidant (Ahmed *et al*., 2015; Yang *et al*., 2019) and antithrombotic (Sabbione *et al*., 2016) activities, the search for BAPs with antihypertensive activity has increased (Amorim *et al*., 2019; Guo *et al*., 2019).

Hypertension, one of the major health issues, is a causative factor of serious diseases such as kidney and cardiovascular diseases (Admassu *et al*., 2018). The endothelium of the kidney and the capillaries of the lungs both express angiotensin I-converting enzyme (ACE) (Ahmad *et al*., 2023). ACE has an important part in increasing blood pressure by converting angiotensin I to angiotensin II which is an active vasoconstrictor. On the other hand, reduction of ACE activity prevents the production of angiotensin II which lowers blood pressure and causes vasodilation (Wu *et al*., 2020). Consequently, various ACE inhibitory drugs are currently used to treat hypertension (Daskaya-Dikmen *et al*., 2017). However, it is possible to recognise the adverse impacts of synthetic drugs such as headaches, dry caught, allergic symptoms, taste disturbances and drug–drug interactions (Paiva *et al*., 2016; Villadóniga & Cantera, 2019). Hence, the search for natural alternatives to drugs responsible for

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inhibiting ACE activity has become an important issue, especially for the pharmaceutical industry (Ibrahim *et al*., 2017; Zheng *et al*., 2020).

Bioactive peptides exhibiting ACE-I activity from different sources have been shown in recent studies, including casein (Tu *et al*., 2018), fish (Abachi *et al*., 2019), soybean protein isolate (Xu *et al*., 2021) and black soybean (Li *et al*., 2018). In addition to variations arising from sources, there are different strategies for producing these BAPs. While enzymatic hydrolysis is a widely preferred method (Sornwatana *et al*., 2015), fermentation is another strategy with the advantage of being inexpensive (Undhad Trupti *et al*., 2021). While microbial fermentation with culture showing proteolytic activity is fundamental for releasing BAPs, lactic acid bacteria are involved in most studies (Shu *et al*., 2017; Daliri *et al*., 2018; Mazorra-Manzano *et al*., 2020; Undhad Trupti *et al*., 2021). However, studies focused on yeast fermentation to produce BAPs with ACE inhibitory activity are limited (Li *et al*., 2015).

Yamadazyma spp., about which there are few studies, have been isolated from different sources, including *Yamadazyma triangularis* from dairy environments (Agarbati *et al*., 2021) and *Yamadazyma terventina* from olive oil (Ciafardini *et al*., 2013). In addition, the proteolytic activity of *Yamadazyma* spp. has been shown in some studies (Cai *et al*., 2022), and even cold-active protease-producing strains have been isolated (Daskaya-Dikmen *et al*., 2018). However, the potential of *Yamadazyma* spp. fermentation to release BAPs to inhibit ACE activity remains unknown.

In this context, this research was undertaken to determine the proteolytic activity of six different *Yamadazyma* spp. isolates at two different temperatures, and to investigate the impact of *Yamadazyma* spp. fermentation on non-fat milk protein and the capacity of released BAPs in terms of ACE inhibitory activity. Additionally, it is planned to contribute to the literature by investigating the potential effects of microbial combination on bioactivity.

Materials and methods

Materials

Hippurl-1-histidyl-l-leucine (HHL, Sigma-Aldrich, USA) and ACE from rabbit lung (Sigma-Aldrich, USA) were supplied. All other chemicals used were of analytical grade and commercially available.

Six previously isolated yeast strains, namely *Yamadazyma* spp. BO10, BO13-2, BO15-2, BO4, B514-1 and B514-2 from Bolu Mountain in Türkiye, were used in the study (Daskaya-Dikmen *et al*., 2018). Their stock cultures at −80 °C in 15% glycerol (v/v) were maintained by propagation through sub-culturing.

Methods

Proteolytic activities of the isolates

Tryptic soy broth (TSB; Merck, Darmstadt, Germany) was used to subculture the all isolates, and incubation was carried out at 25 °C for 48 h. Then, 10 μL of single or combined yeast aliquot in the concentration of 4 McFarland was inoculated on the basal medium supplemented with non-fat milk (g/L, yeast extract 1; $MgSO_4.7H_2O$, KH_2PO_4, 10.1; $CaCl_2.H_2O$, 0.05; NaCl, 5; $NaCO_3$, 1; and agar, 18 in 850 mL distilled water and 150 mL non-fat milk) as a single point (Yadav *et al*., 2016). The diameter of the hydrolysis zone which occurs due to precipitation around the colony at the end of incubation at 15 and 25 °C for 24, 48 and 72 h was measured to determine the proteolytic activities of the isolates.

Pz index value was calculated to evaluate the proteolytic activity, which is the ratio of the total colony diameter to the total precipitation halo plus the total colony diameter. The ranges used to assess proteolytic were high, Pz < 0.40; medium, Pz = 0.41–0.60; low, Pz = 0.61–0.80; very low, Pz = 0.81–0.99; and none, Pz = 1 (Galán-Ladero *et al*., 2010).

Microbial fermentation

Non-fat milk as a protein source was fermented by three yeast strains separately and their double and triple combinations: BO10 & B514-1, BO10 & BO13-2, B514-1 & BO13-2 and BO10 & B514-1 & BO13-2. Yeast suspension (2 mL) at a concentration of 4 McFarland was inoculated into the medium and incubated at 25 °C at 150 rpm for 48 h. Samples were collected at each 24-h interval, and the supernatant was separated (10 000 ***g***, 20 min) and stored at −40 °C for further analysis.

ACE-I activity of the isolates

Firstly, samples were prepared to determine the ACE-I activity (Chaves-López *et al*., 2012; Rayaprolu *et al*., 2015; Aissaoui *et al*., 2017). Briefly, 100 mM borate buffer solution was added to the collected supernatants from microbial fermented samples (1:4 v/v). The enzymatic reaction was initiated by adding ACE solution (20 μL) into 250 μL of supernatant and buffer mixture. After the incubation at 37 °C for 60 min, the termination of the reaction was performed by adding 1 N HCl (250 μL). Ethyl acetate (700 μL) was added to extract the hippuric acid (HA), and the mixture was centrifuged at 4000 ***g*** for 10 min. Then, 700 μL of the upper part was removed, and ethyl acetate was evaporated by heating at 100 °C for 60 min. Before analysis by HPLC, 700 μL water was added to the samples.

The ACE-I activity of samples was measured by using HPLC (Agilent Technologies 1100 system) with a modified method performed by Liu *et al*. (2010)

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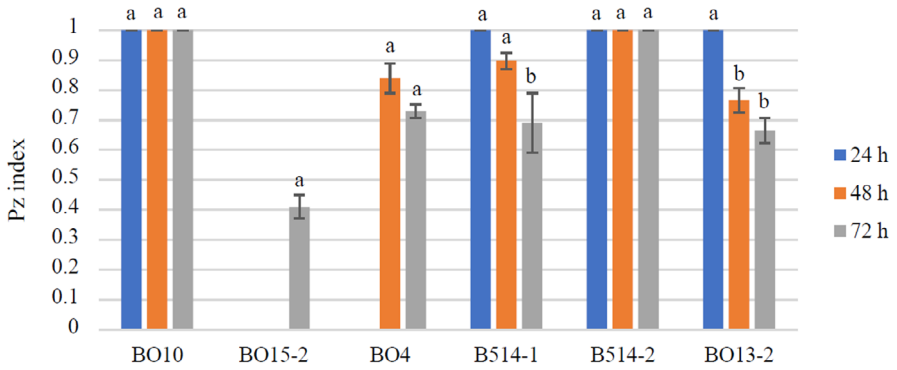

Figure 1 Secretion of protease by isolates at 15 °C. Activity is expressed as the Pz index (mean ± standard deviation). [a, b]Values for the same isolates with different superscript small letters are significantly different ($P < 0.05$).

based on the determination of HA released by ACE from the substrate of hippuryl-L-histidyl-L-leucine (HHL). Briefly, samples were analysed using a C18 column (Supelco, USA, 250 mm × 4.6 mm, 5 µm). The detection of HA was done with a DAD detector (Agilent G1315D) at 228 nm. The isocratic flow with mobile phases of acetonitrile (A) and HPLC grade water including 0.5% trifluoroacetic acid (B) at 25:75 was used. The ACE-I activity of the supernatants was determined by the following equation:

$$\text{ACE}-\text{I activity } (\%) = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is the concentration of HA from the reaction control, in which the mixture contains the same volume of the buffer solution instead of the ACE inhibitor sample, and A_s is the concentration of HA from the reaction in the presence of both ACE and its inhibitor.

Then, IC_{50} values, the concentration of supernatants for 50% inhibition (Gulcin & Alwasel, 2022), were calculated. The peptide concentrations of supernatants were calculated using the Lowry method (Lowry *et al.*, 1951).

Partial purification of ACE inhibitory peptides

The supernatants resulting in higher ACE-I activity were partially fractionated through ultrafiltration membranes in a pierce protein concentrator with 10 kDa molecular weight cutoff (Thermo Scientific, No: 88528). Samples (15 mL) placed in the concentrator sample chamber were centrifuged (8000 ***g***, 10 min, 4 °C), and collected fractions were stored at −40 °C until further analysis. ACE inhibitory activity of all fractions was determined as previously mentioned in ACE-I activity of the isolates.

Statistical analysis

All experiments were performed at least in duplicate, and the resulting data were analysed by Minitab Statistical Software (Version 19). One-way analysis of variance (ANOVA) and multivariance tests were performed at 0.05 significance level. Mean ± standard deviation was used to express the results.

Results and discussion

Proteolytic activities of the isolates

The ability of isolates to secrete extracellular protease at two different temperatures (15 and 25 °C) was analysed by measuring the precipitation area and colony diameter on a non-fat milk medium. Figures 1 and 2 show the qualitative results and calculated Pz index values. As seen in Fig. 1, at 15 °C, Pz values of both *Yamadazyma* spp. BO10 and B514-2 were equal to 1 during incubation which indicates no protease activity. Besides, the growth of *Yamadazyma* spp. BO15-2 and BO4 at 15 °C was lower than other isolates. The lowest Pz value was found for *Yamadazyma* spp. BO15-2 (Pz = 0.410 ± 0.039) at 72 h incubation, followed by *Yamadazyma* spp. BO13-2 (Pz = 0.665 ± 0.041).

On the other hand, at 25 °C, all isolates were protease positive, and incubation time was an effective factor in the proteolytic activity of the isolates, which generally increased as the incubation time increased (Fig. 2). Compared to the results at 15 °C, *Yamadazyma* spp. B514-2 showed no growth at 25 °C. While the growth of *Yamadazyma* spp. BO15-2 and BO4 was observed during all incubation periods, the proteolytic activity of *Yamadazyma* spp. BO15-2 was assessed to be lower due to greater Pz value. Among incubation at 15 and 25 °C, the proteolytic activities of isolates were found higher at 25 °C, and several combinations of three different *Yamadazyma* spp. isolates (BO10, BO13-2 and B514-1) to produce protease were tested at 25 °C. Similar to the study of Nicodème *et al.* (2005), temperature has an observable impact on growth and proteolytic activity. Besides, there was generally a statistically significant difference in

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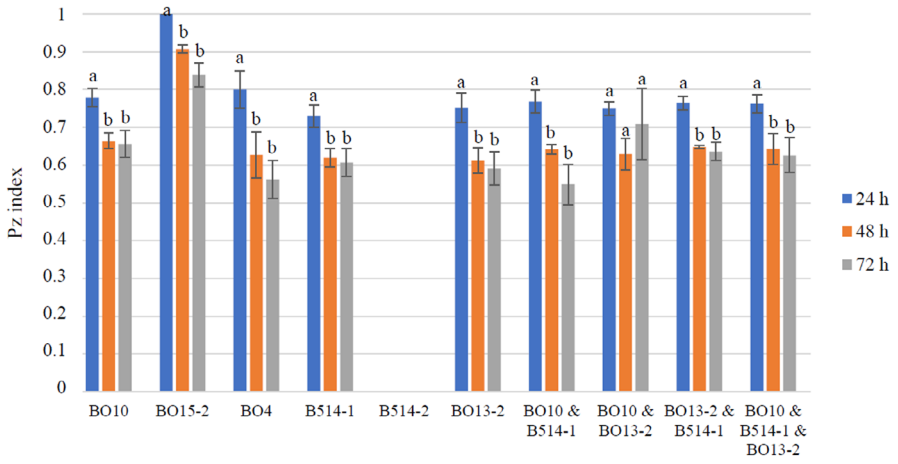

Figure 2 Secretion of protease by isolates or in combination at 25 °C. Activity is expressed as the Pz index (mean ± standard deviation). [a, b]Values for the same isolates with different superscript small letters are significantly different ($P < 0.05$).

proteolytic activity between the 24 and 48 h incubation periods, except for the combination of BO10 and BO13-2. However, there was no significant difference between 48 and 72 h.

As stated by Galán-Ladero *et al.* (2010), the enzymatic activity increases as the Pz value decreases. At the end of 24 h of incubation, the lowest Pz value was in *Yamadazyma* spp. B514-1 (Pz = 0.730), whereas all isolates were evaluated in the same Pz value enzyme activity range. This range appears to be similar to other incubation times. Although there are different yeast cultures with higher proteolytic activity based on the Pz index evaluation (Fernández-Pacheco *et al.*, 2021), very limited studies about *Yamadazyma* spp. have been found in the literature. The proteolytic activity of *Yamadazyma terventina* and *Yamadazyma triangularis* was detected by Ciafardini *et al.* (2013) and Cai *et al.* (2022) respectively. On the other hand, *Yamadazyma triangularis* exhibited no proteolytic activity (Agarbati *et al.*, 2021), which suggests that various properties, including the isolation source, may differ in the activity of the culture and the metabolites it produces. In the case of Daskaya-Dikmen *et al.* (2018), who were studied at 15 °C for cold-active protease enzyme production ability of the same isolates, there was no difference in protease-producing isolates with increasing incubation, but the activity increased with time. As supported by these findings, 24 and 48 h of incubation time were selected for the determination of ACE-I activity of single and mixed isolates.

ACE inhibitory activity of the isolates

The ACE-I activity in the samples fermented with selected single *Yamadazyma* spp. isolates or their combinations was determined by the chromatographic method based on the principle of conversion of HA to HHL (Table 1). The ACE-I activity of the isolates generally decreased with an increase in incubation time, except for the combination of *Yamadazyma* spp. BO10 and *Yamadazyma* spp. BO13-2, similar to that reported by Shu *et al.* (2015). They emphasised the possibility that ACE-I peptides would be degraded by the enzymes produced along with increasing titration acidity after a certain incubation period. In accordance with the current study, there was generally a decline in ACE-I by increasing storage, which was associated with decreasing pH (Şanli *et al.*, 2018). The activity of low-molecular-weight peptides released by the possible degradation of ACE-I peptides may be lower (Nielsen *et al.*, 2009; Şanli *et al.*, 2018). It is likely to attribute the degradation to differences in the specificity of the enzymes produced by microorganisms (Donkor *et al.*, 2007).

Table 1 ACE-I activity of single and combined *Yamadazyma* spp. isolates

	ACE-I activity (%)	
Isolate code	**24 h**	**48 h**
BO10	67.85 ± 9.12^{ab}	n.d.
B514-1	54.67 ± 8.74^{b}	–
BO13-2	80.12 ± 1.62^{ab}	–
BO10 & B514-1	92.51 ± 15.0^{a}	42.86 ± 33.51^{b}
BO10 & BO13-2	56.14 ± 32.56^{b}	76.00 ± 1.69^{a}
B514-1 & BO13-2	75.77 ± 21.02^{ab}	61.13 ± 9.67^{ab}
BO10 & B514-1 & BO13-2	71.29 ± 10.82^{ab}	66.59 ± 7.96^{ab}

Values within the same column with different superscript small letters "$^{a, b}$" are significantly different ($P < 0.05$).
n.d., not detected.

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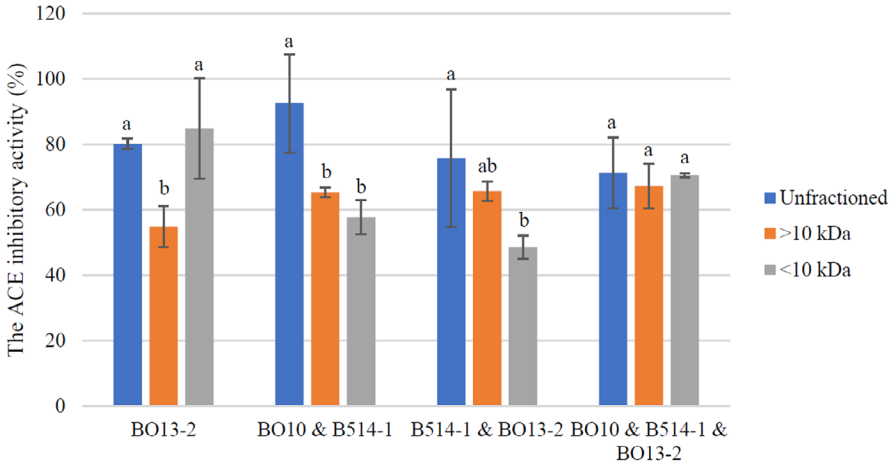

Figure 3 ACE inhibition activity of yeast isolates after ultrafiltration. [a, b]Values for the same isolates with different superscript small letters are significantly different ($P < 0.05$).

While *Yamadazyma* spp. BO10 and *Yamadazyma* spp. B514-1 individually presented low ACE-I activity, their combination was found to be statistically different ($P < 0.05$) from the others with the greatest activity in the same incubation time. Similarly, ACE-I activity was positively impacted by the culture combination (Loghman *et al.*, 2022). However, there may be differences between cultures, and the combination may not always result in a synergistic effect. Despite the few studies discussing the synergistic effect of yeasts, the lactic acid bacteria combination was indicated as a remarkable option for enhanced ACE-I activity (Olvera-Rosales *et al.*, 2023).

All single isolates and their combinations were found to have remarkable ACE-I activity at 24 h incubation in the range of 54–92%. On the other hand, no activity was detected after 48 h for single isolates. Since there are not many publications about the ACE-I activity of *Yamadazyma* spp., comparison at the isolate level is not possible. However, in the study by Abachi *et al.* (2019) examining the ACE-I activity of various lactic acid bacteria isolates, the results varied between 5% and 74%. In another study conducted using different yeast isolates, although not all species or isolates showed activity under the studied conditions, positive results were found to be 9–80% (Chaves-López *et al.*, 2012).

Abd El-Fattah *et al.* (2017) have demonstrated that ACE-I activity and proteolytic activity are positively correlated. Despite this fact, there are also studies that have concluded no correlation between proteolytic activity and ACE-I activity, similar to this study (Ramchandran & Shah, 2010). Similarly, in the study of Donkor *et al.* (2005), it was determined that *Lactobacillus acidophilus*, which has high proteolysis, had low ACE-I activity. In the current study, although the range of protease enzyme activity evaluation based on the Pz index was similar for all isolates and combinations, no positive correlation was observed in their ACE-I activity for some cases. *Yamadazyma* spp. BO10, B514-1 and the combination of BO10 and B514-1 had higher proteolytic activity (lower Pz index) at 48 h, and the maximum ACE-I activity was seen after 24 h of incubation under the performed conditions.

Partial purification of ACE inhibitory peptides

As seen in Table 1, the samples with the greatest ACE-I activity (BO13-2, BO10 & B514-1, B514-1 & BO13-2 and BO10 & B514-1 & BO13-2) were partially purified by ultrafiltration. ACE-I activity in samples separated to be smaller or larger than 10 kDa was evaluated (Fig. 3). Generally, the effect of ultrafiltration on ACE-I was found to be significant, and samples often had lower ACE-I activity than unfractionated samples. However, no statistically significant difference was observed in the partially purified fractions ($P > 0.05$). This concludes that an additional purification process or filtration with a lower molecular weight may be required to obtain increased ACE-I activity. It has been previously reported that the fraction of <5 kDa exhibited higher ACE-I as contrary to unfractionated or >5 kDa fractions (Ko *et al.*, 2012). Similarly, *Ulva rigida* protein hydrolysate fractionation by ultrafiltration resulted in the strongest ACE-I activity for fractions with molecular weights lower than 1 kDa (Paiva *et al.*, 2016). In accordance with these results, it was also stated that the low-molecular-weight fractions exhibited more ACE-I activity than the higher-molecular-weight fractions (Jung *et al.*, 2006). In addition, it has been

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shown that fractions between 5 and 10 kDa had lower activity than unfractionated fractions (Mirzaei *et al*., 2018).

The concentration of protein in the collected supernatants required to inhibit 50% of the ACE activity (IC_{50}) was also determined for the combination of yeast isolates. The IC_{50} values of the samples were found higher than IC_{50} values of the active substances commonly used in antihypertensive drugs such as captopril (IC_{50} value 1.3×10^{-6} mg/mL). The combination of BO10 and BO13-2 (IC_{50} value 0.92 mg/mL) was lower than the other studied combinations, BO10 and B514-1 (IC_{50} value 2.10 mg/mL) and B514-1 and BO13-2 (IC_{50} value 2.46 mg/mL). It is possible to observe variation in the results of IC_{50}, especially due to the source, conducted method and purification method used. According to Bao & Chi (2016), IC_{50} values for different strains of *Lactobacillus casei* ranged from 0.89 to 4.31 mg/mL. Ko *et al*. (2012) emphasised the impact of purification on ACE-I activity and its effect on IC_{50} value. With the various purification methods, notable variations in the IC_{50} values were detected. While the value of 0.06 mg/mL was detected by RP-HPLC, the IC_{50} value in the sample obtained by an ultrafiltration method with a cutoff value of <5 kDa was found to be 0.89 mg/mL, which is approximately fifteen times higher than the value of RP-HPLC.

Conclusion

The ACE-I activity of previously isolated *Yamadazyma* spp. from different sources and their synergistic effects were evaluated. For this purpose, non-fat milk was used as a protein source, and fermented by isolates with a certain level of proteolytic activity. The BAPs released had ACE-I activity at values comparable to those reported in the literature. Moreover, the study has gained significant originality by investigating the impact of combining isolates on bioactivity. Remarkable results in terms of bioactivity have been obtained as a result of the synergistic effects demonstrated by isolates. The combination of the BO10 and BO13-2, which possessed ACE-I potential, exhibited an IC_{50} value of 0.92 mg/mL. Ultrafiltration was also performed to evaluate the effect of partial purification, with the potential to increase ACE inhibitor activities through further purification. Furthermore, these findings support the idea that employing yeasts in product fermentation might help benefit from their functional and health-promoting characteristics, which have not received much research. In addition, biological effects as a result of the consumption of BAPs obtained by advanced purification or fermented milk products in which these BAPs are released in order to be presented as an alternative to the existing high blood pressure drugs available in the market are potentially the subject of the future studies.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

Ethics approval was not required for this research.

Author contributions

Dilara Devecioglu: Methodology; investigation; writing – original draft. **Busra Sarıakcalı:** Methodology; investigation; writing – original draft. **Betul Orhan:** Methodology; investigation. **Ceren Daskaya-Dikmen:** Investigation; conceptualization. **Beraat Ozcelik:** Investigation; conceptualization; supervision. **Funda Karbancioglu-Guler:** Investigation; conceptualization; resources; funding acquisition; writing – review and editing; supervision.

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Peer review

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article. Raw data that support the findings of this study are available from corresponding author upon reasonable request.

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