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Biochemical Properties of Oregano β-Glucosidase Supports *Origanum Onites* L. as a Feed Additive

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ABSTRACT

Background: Feed additives are used for different purposes and include different substances. Recently, the feed additive potential of aromatic plants has been frequently investigated. β-glucosidases are used as feed additive enzymes to increase nutritional value. This study was carried out to determine the biochemical properties of *Oregano onites* L. β-glucosidase in terms of expected biochemical properties from feed additive enzymes.

Methods: Oregano β-glucosidase was purified by hydrophobic interaction chromatography. The purified enzyme was checked the purity on SDS-PAGE. The biochemical properties (optimal pH and temperature, thermal stability, glucose and alcohol tolerance) of the purified enzyme were determined using *para*-Nitrophenyl-β-D-glucopyranoside as a substrate.

Result: *Origanum onites* L. β-glucosidase was purified to electrophoretic homojenity with 23.15-fold with a yield of 8.2% and it was visualised as a single band at 65.7 kDa on SDS-PAGE. The enzyme had maximum activity at pH 4.0 and 45°C and retained over 50% activity at pH 4-7. The enzyme maintained up to 50% of its activity after 60 minutes of incubation at 40°C, 50°C, and 60°C. The purified enzyme showed a high tolerance to glucose and was also found to be tolerant to alcohol. The enzyme's biochemical properties similar to exogenous enzymes recommended as feed additives. Therefore, the results of this study supports the use of oregano as a feed additive.

Key words: Aromatic plant, β-glucosidase, Enzyme characterisation, Feed additive, *Origanum onites* L.

INTRODUCTION

Feed additives are used in animal nutrition for different purposes, such as enhancing feed quality, regulating the digestive system, increasing meat quality and yield and supporting animal health (Pandey *et al*., 2019). However, since the adverse effects of some additives on human and animal health have been determined over time, researchers have turned to alternative additives. In the European Union, it has been prohibited to give antibiotics as a feed additive to ruminant animals since 2003. (Directive 1831/2003/CEE, European Commission, 2003) (Kozłowska *et al*., 2020). Especially after this decision, research on natural product feed additives has increased even more. Medicinal and aromatic plants contain intense phenolic substances, which are antimicrobial, antioxidant, anti-inflammatory and antimethanogenic due to their effects additive potentials have been frequently investigated (Pandey *et al*., 2019). Different types of thyme have been investigated as feed additives and many positive effects on animal health have been revealed (Paraskevakis, 2018; Stivanin *et al*., 2019; Vizzotto *et al*., 2021).

In addition to natural plant additives, enzymes produced by biotechnological methods are also included in food and feed additives (Pandey *et al*., 2019). β-glucosidases hydrolyse glycosidic bonded compounds and are used as additives to increase nutritional quality in food and feeds (Ketudat Cairns and Esen, 2010). They have been widely used to convert inactive and glycosidic-linked isoflavones to active aglycones, especially in soybean products (Cao *et al*., 2018; Delgado *et al*., 2021). When adding enzymes produced in expression systems to foods and feeds, there are doubts about organism residue (Deckers *et al*., 2020). From this point of view, this study is aimed to purify the β-glucosidase of Turkish oregano used as a herbal feed additive to determine its biochemical properties and to compare it with β-glucosidases produced by biotechnological methods.

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MATERIALS AND METHODS

Plant samples and chemicals

Turkish oregano (*Origanum onites* L.) was collected from Izmir, Turkey, in May-June. Sepharose 4B, L-Tyrosine, 1-Naphtylamine and Substrates *para*-Nitrophenyl β-D-glucopyranoside (*p*NPG) and *ortho*-Nitrophenyl β-D-

glucopyranoside (*o*NPG) were supplied by Sigma Aldrich (from the USA). Other chemicals were purchased from Merck. This study was partially carried out at Balikesir University Veterinary Faculty Biochemistry Laboratory in 2016 and completed at Bursa Uludağ University Veterinary Faculty Biochemistry Laboratory in 2022.

The enzyme extraction

When extracting enzymes from plants with high oil content, acetone powder is preferred to eliminate the oil (Romero-Segura *et al*., 2009). Acetone powder was prepared using all parts of the plant for extraction. For this, 15 g of oregano was homogenised with 200 mL of cold acetone (-20°C) with a kitchen blender for 2 minutes on ice and this process was done twice. The resulting pellet was dried at room temperature and stored at -20°C. For extraction, 0.5 g of oregano acetone powder was homogenised with 25 mL of extraction buffer (0.1 M Tris Buffer pH 8.0 with 0.1 M NaCl and 0.02% NaN3) with a homogeniser (Stuart SHM1) on ice for 2 minutes. The homogenate was centrifuged at 15000 rpm for 30 minutes (Sigma, 3k30 centrifuge) and the supernatant was used as the crude extract.

Purification of the β-glucosidase from *Origanum onites* L.

The enzyme purification process was carried out in two stages, as in the Kara *et al*. (2011) method. Firstly, the precipitation of salt was applied for partial purification with 20-60% ammonium sulfate concentration (appropriate salt concentration was determined as a result of experiments). The pellet, which was precipitated with salt, was dissolved in sodium phosphate buffer (minimum volume, 50 mM, pH 6.8) and then applied to the hydrophobic coloumn (Sepharose 4B-L-tyrosine-1-naphthylamine hydrophobic gel, 1×10 cm). Before sample application, the column was equilibrated with the sodium phosphate buffer containing 1 M ammonium sulfate (50 mM, pH 6.8 sodium phosphate buffer containing 1 M ammonium sulfate). After applying the sample, a 1.5-0 M ammonium sulfate salt gradient was created. Elution was performed at 0.5 mL/min and eluents were taken in 2 mL tubes. Qualitative protein assay (Absorbance 280 nm) and enzyme activity assay were performed on each collected eluent. The eluents with high enzyme activity were combined and this solution used as a pure enzyme in experiments.

SDS-PAGE and zymogram assay

To control the purity of the purified enzyme and to determine its molecular weight, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). The SDS-PAGE technique prepared the stacking and separation gels as batch gels at 3% and 10% acrylamide concentration, respectively. The proteins were run at 120 volts of electric current (Mini Protean Tetra Cell Bio-Rad) until they migrated 0.5 cm from the base of the separation gel (10%). Protein bands were stained with Coomassie brilliant blue dye (R-250) (Sigma), the excess dye in the gel was removed with a suitable solvent and the bands were visualised. Molecular weight marker (Thermo Fisher, 26610) 116.0kDa, 66.2 kDa, 45.0 kDa, 35.0 kDa, 25.0 kDa, 18.4 kDa and 14.4 kDa, was used.

Enzyme assay and protein determination

The enzyme activity was determined spectrophotometrically. 5 mM *p/o*NPG substrate and enzyme solution were put into the well of a 96-well plate and incubated at 37°C for 30 minutes. After incubation, the reaction was stopped with 0.5 M Na_2CO_3. In the spectrophotometer (BioTech, Epoch), readings were done at 405 nm for the determination of *p*-nitrophenol (*p*NP) and 420 nm for the determination of *o*-nitrophenol (*o*NP). Absorbance values were converted to EU values using p/oNP standard plots. One Enzyme Unit (EU) is defined as µmol of *p/o*NP occurring per minute.

The Warburg- Christian Method performed qualitative protein determination based on absorbance determination at 280 nm and the quantitative protein determination was determined by the Lowry Method.

Determination of optimum pH and temperature

The optimum pH of the purified oregano β-glucosidase was determined at different pHs in the 2.30-12.30 intervals (50 mM Na-Ac buffer was prepared). Enzyme activity (EU) was found at each pH value. The enzyme activity was found and incubated at different values at 35-65°C to determine the optimum temperature.

Determination of thermal stability

The purified enzyme was incubated for 60 minutes in a water bath at 40-50-60°C to determine the thermal stability. The reaction was carried out in pH 5.5 acetate buffer. Enzyme activity at zero minutes was accepted as 100% and the enzyme activity at every 5 minutes was determined. The activity values found were calculated as relative activity and plotted with GraphPad Prism 6.0 software. The experiments were performed in duplicate and the error bars in the graph show the standard deviation.

Glucose and alcohol tolerance analysis

The effect of glucose and ethanol on the purified enzyme activity was determined at different glucose (ranging from 100 to 1100 mM) and ethanol (0-30%, v/v) concentrations in the reaction medium under standard activity assay conditions. *p*NPG was used as a substrate in the reactions. Enzyme activity without added glucose/ethanol was represented as 100%, relative activities were calculated for each glucose or ethanol concentration and graphs were drawn. Experiments were repeated in duplicate and error bars show standard deviation values.

Substrate specificity

Para/ortho-nitrophenyl β-D-glucopyranoside (*p/o*NPG) are commercial substrates of the enzyme prepared as 5 mM and enzyme activities at 37°C in pH 5.5 Na-Ac buffer and determined as described. The kinetic parameters (V_{max} and K_m) of the purified β-glucosidase were determined by Graph Pad Prism 9.0 software according to the Michaelis-Menten equation by measuring the enzyme activity at different concentrations of the *p/o*NPG substrates.

RESULTS AND DISCUSSION

Enzyme purification

Oregano β-glucosidase was purified by a two-step process. The salt precipitation removed most of the contaminants and the total amount of protein decreased from 117.3 mg to 13.5 mg (Table 1).

The elution step of hydrophobic interaction chromatography is plotted in Fig 1. At the end of the chromatography, the enzyme activity was preserved close to 10% and the total protein amount decreased from 13.5 mg to 0.49 mg according to the ammonium sulfate precipitation step. At the end of all purification processes, Oregano β-glucosidase had a specific activity of 16,16 U/mg and was purified 23.2 times in a 9.58% yield (Table 1).

It was seen as a single band on SDS-PAGE. The enzyme was purified to electrophoretic homogeneity (Fig 2A) and its estimated subunit molecular mass was calculated as approximately 65.7 kDa using the Log molecular weight-Relative flow plot given in Fig 2B.

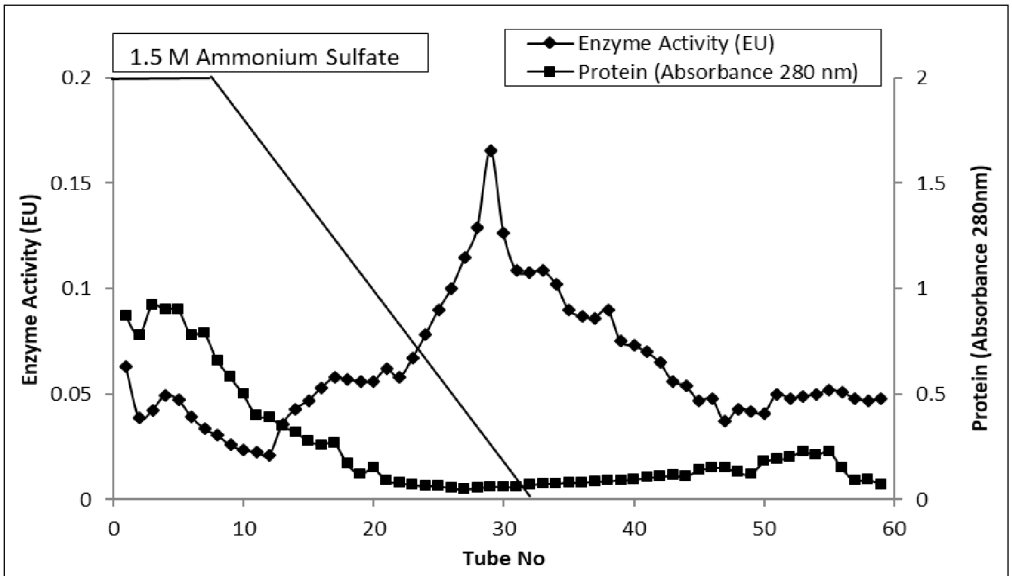
In this study, a β-glucosidase was purified to electrophoretic homogeneity from the Turkish oregano plant and was characterised for the first time. The enzyme yield was higher than β-glucosidases purified from *Aspergillus flavus* (Chen *et al*., 2019). The low number of purification steps was influential in the emergence of this result.

β-glucosidases are isolated from many different sources for use as food and feed additives. The purified oregano β-glucosidase, with a specific activity of 16.2 U mg^{-1}, is close to other enzymes, such as specific activities of *Aspergillus flavus* (Chen *et al*., 2019) and *Alicyclobacillus herbarius* (Delgado *et al*., 2021) β-glucosidases, which hydrolyse soybean isoflavone glycosides to increase their nutritional value. Moreover, the specific activity of the oregano β-glucosidase is considerably higher than that of the recombinant *Thermotoga maritima* β-glucosidase A (BglA) (Xue *et al*., 2009), which catalyses the hydrolysis of soy isoflavone glycosides. This result indicates that the potential of oregano to hydrolyze glycosidic compounds found in soybean. Therefore, it will be more innocent to use natural additives in animal feeds instead of an exogenous enzyme produced by biotechnological methods.

The purified enzyme, with a subunit molecular weight of 65.7 kDa, showed similarity to olive (Kara *et al*., 2011; Romero-Segura *et al*., 2009) and peppermint (Kara *et al*., 2022) β-glucosidases and is close to β-glucosidase isolated from the Mangrove reserve metagenome that 66 kDa (Li *et al*., 2012). β-glucosidases are included in the Glycoside

Table 1: Enzyme purification steps.

Phase	Total protein amount (mg)	Specific activity of the enzyme (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	117.3	0.70	100	1
Salt precipitation	13.5	0.82	13.5	1.2
Hydrophobic interaction chromatography	0.49	16.16	9.6	23.2



Protein concentrations and enzyme activities of the fractions collected in the elution step. The amount of protein is expressed as absorbances measured at 280 nm, and the enzyme activity refers to values determined using the *p*NPG substrate.

Fig 1: Purification of the enzyme by the method of hydrophobic interaction chromatography.

Hydrolase Families GH1, GH3, GH5, GH9 and GH30 and mostly GH1 family proteins have been characterised in plants (Ketudat Cairns and Esen, 2010). The lengths and subunit masses of GH1 enzymes vary depending on the presence of auxiliary domains and redundant. The subunit molecular mass of the purified enzyme from Turkish oregano was evaluated as compatible with GH1 β-glucosidases.

Substrate specificity of the oregano β-glucosidase

The kinetic parameters of the purified enzyme were determined using *p*NPG and *o*NPG substrates. Michaelis-Menten plots drawn using GraphPad Prism 9.0 software (Graphs are not shown) and the kinetic constants K_m and V_{max} are the values calculated by GraphPad Prism. The enzyme's K_m values against *p/o*NPG substrates were 0.68 and 1.76 mM, respectively, indicating a higher affinity for *p*NPG than for *o*NPG. V_{max} values were 1855 and 3493 μM.min^{-1} for *p/o*NPG, respectively.

Although β-glucosidases exhibit a high diversity of substrate specificity, it has often been reported to have a greater affinity for *p*NPG than for *o*NPG (Ketudat Cairns and Esen, 2010). This report is in line with this study. The K_m value found for the enzyme as a substrate *p*NPG is considerably lower than β-glucosidase purified from *Aspergillus terreus* (Yan *et al*., 2016), which catalyses soybean isoflavone hydrolysis with high efficiency and is recommended as an animal feed additive. The Vmax value of the purified enzyme, 1855 μM.min^{-1} for the *p*NPG substrate, is considerably higher than the V_{max} value of Chayote β-glucosidase isoform II (Cruz *et al*., 2020) and *Brassica oleracea* β-glucosidase (Besic *et al*., 2017) against the same substrate. According to these results, it can be said that the enzyme's catalysis ability is similar to the enzymes recommended for soybean hydrolysis.

Effect of pH and temperature on the purified enzyme

The optimum pH and temperatures of β-Glucosidases differ depending on their sources. Most β-glucosidases have an optimum pH between 4 and 7.5 and are stable in a pH range of 4 to 9 (Ketudat Cairns and Esen, 2010). Turkish oregano β-glucosidase exhibited the highest activity at pH 4.0 and was stable, maintaining its activity above 50% in the pH 4-7 range (Fig 3A). The pH range in which the enzyme showed maximum activity was in the acidic pH range, but the activity was deficient below the pH value of 3.0. The enzyme activity decreased steadily until pH 8.0 to 40% remaining activity levels. After 9.0, this value is around 10% (Fig 3A). A β-glucosidase from *Talaromyce leycettanus*, which catalyses the hydrolysis of soybean isoflavone glycosides, showed optimum activity at pH 4.5 and retained most of its activity over a wide pH range (pH 3.0-9.0) (Li *et al*. 2018). The oregano β-glucosidase is very similar to the enzyme characterised by Li *et al*. (2018), as it can maintain its activity in the pH optima of 4.0 and pH 4.0-7.0 range. β-glucosidases recommended for use as additives in animal feeds should be resistant to pH 4.0-6.5. Because the enzyme is exposed to gastrointestinal pH before showing its catalytic effect in the gut of monogastric (Peng *et al*., 2014) and the mean pH values of the porcine stomach and small intestine are 4.4 and 6.1-6.7, respectively (Yan *et al*., 2016). In ruminants, the rumen's pH is acidic due to fermentation, ranging from 5.5 to 7.0 (Erdman, 1988). The fact that the oregano β-glucosidase maintains its activity at these pHs provides support for using Turkish oregano as an animal feed additive.

The purified oregano β-glucosidase showed maximum activity at 45°C and its residual activity at 45-55°C was >50% (Fig 3B). The enzyme maintained its activity above 50% for 60 minutes at 40, 50 and 60°C (Fig 3C). Mesophilic β-glucosidases exhibit the highest activity at 30-65°C (Ketudat Cairns and Esen, 2010) and the purified oregano β-glucosidase can also be considered mesophilic. The optimum activity of β-glucosidase, isolated from the mangrove soil metagenomic library, which hydrolyses soybean isoflavone glycosides at a high rate, is 40°C (Li *et al*., 2012). The purified oregano β-glucosidase is similar to GH1

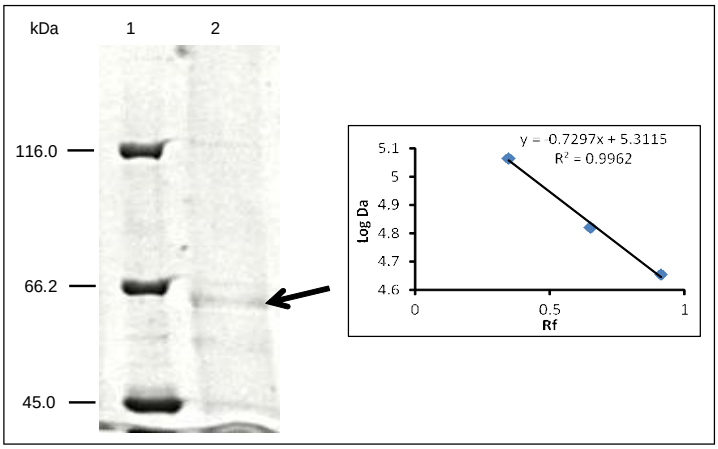


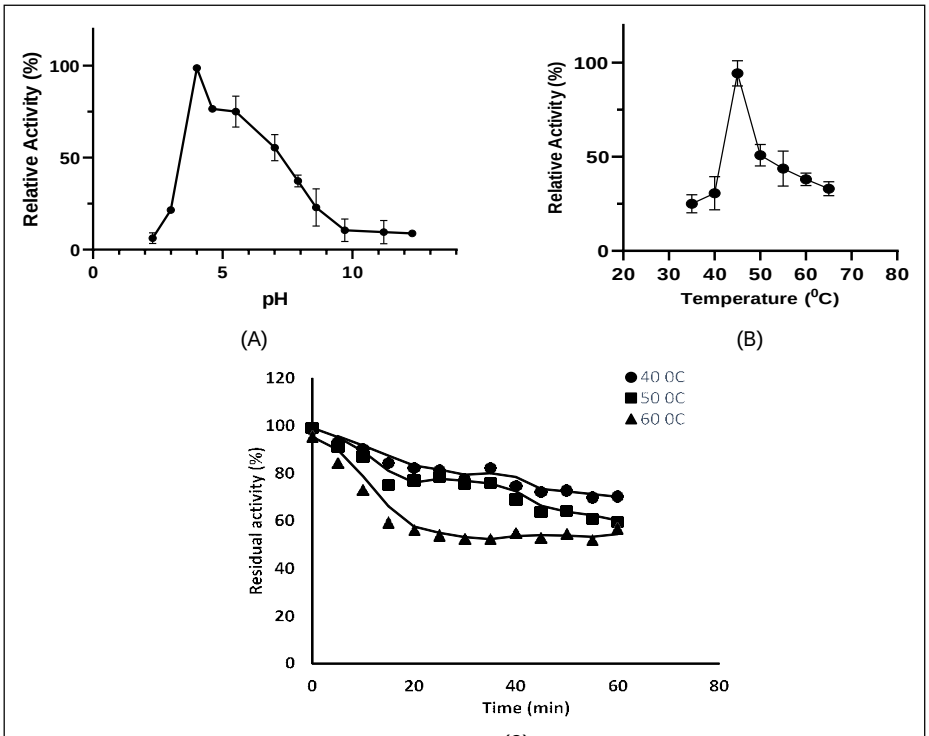
Fig 2: (A) SDS-PAGE analysis of the purified enzyme. The purified enzyme was run in 10% polyacrylamide gel, pH 8.3 and visualized under UV after staining with Coomassie brilliant blue R-250 dye. Lane 1 is protein marker's bants (116 kDa, 66.2 kDa, 45 kDa); Line 2, the purified β-glucosidase from *Turkish oregano* by the hydrophobic interaction chromotography; (B) LogDa-Rf plot used to calculate the molecular weight of the purified enzyme.

family enzymes and the results indicate that the enzyme can catalyze the hydrolysis of soybean isoflavone glycosides.

Another critical advantage of Turkish oregano β-glucosidase is maintaining its activity up to 80% at 40°C. In the study of Li *et al*. (2012), the residual activity of β-glucosidase isolated from soil metagenomic, which was incubated for 30 minutes at 45°C, was 20% and the enzyme we purified seems more capable in terms of this feature. The thermal stability feature of the enzyme encourages the use of Turkish oregano as an animal feed additive.

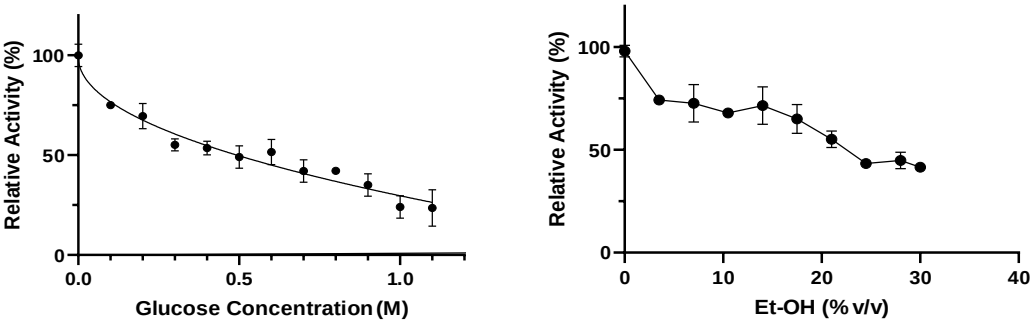
Determination of glucose and ethanol effects

While the glucose concentration in the reaction medium was 0.6 M, the relative activity was 52%. The activity was relatively high at glucose concentrations <0.6 M. Glucose concentration exhibited an average of 35% relative activity in the range of 0.7-1.0 M (Fig 4A). It can be said that the enzyme tolerates up to 1M glucose concentration. Inhibition by glucose of β-glucosidases is one of the crucial problems in many industrial uses, such as improving wine flavour and increasing the nutritional value of food and feed (Kaushal *et al*., 2021). Therefore, β-glucosidases with high glucose tolerance are very valuable. Among the Glycosyl Hydrolases, GH1 family β-glucosidases are ten times to 1000 times more glucose tolerant than those in the GH3 family. In structural analysis, glucose tolerance is related to the active site accessibility of enzymes. The active site of GH1 β-glucosidases has been determined to be deeper and narrower than the enzymes in the GH3 family (de Giuseppe *et al*., 2014). Therefore, considering the presence of glucose in the digestive system, β-glucosidases in the GH1 family would be more suitable as animal feed additives (Kaushal *et al*., 2021). While the relative activity of β-glucosidase belonging to *Trichoderma harzianum*, one of the GH1 family member β-glucosidases, was 30% at 0.8 M glucose concentration (Florindo *et al*., 2017), β-glucosidase from *T. aotearoense* examined by overexpression was 40% (Yang *et al*., 2015). The results of oregano β-glucosidase are consistent with these reports and support that the purified enzyme is similar to GH1 family enzymes and exhibits glucose tolerance properties. Different aromatic plants as feed additives; rumen fermentation, blood enzyme parameters, improving effects on milk and meat yield in ruminants (Paraskevakis, 2018; Vizotto *et al*., 2021), improving digestion, increasing nutrient absorption, antioxidant properties and strengthening the immunity of animals (Zeng *et al*., 2015; Cao *et al*., 2018) have been reported. The glucose tolerance of Oregano β-glucosidase increases the potential of *Origanum onites* L. as a feed additive. β-glucosidase can hydrolyse daidzin, glycitine and genistin glycosides found in soy to flavonoid bioavailable products (Delgado



Average values and standard deviations are represented in the graphs. Error bars show standard deviations (n =3).

Fig 3: Effect of pH (A) and temperature (B) on the purified enzyme activity and the thermal stability (C). Enzyme activity was determined with *p*NPG as a substrate at 37°C.



Average values and standard deviations are represented in the graphs. Enzyme activity was determined by standard activity measurement at 37°C. Error bars show standard deviations (n=3).

Fig 4: Effects of (A) Glucose and (B) Et-OH on the purified enzyme.

et al., 2021). Using oregano as an additive in animal feeds containing soy may be beneficial.

The relative activity of purified β-glucosidase from Turkish oregano in the presence of 10% (v/v) ethanol exhibited high ethanol tolerance of 68% and at 20% ethanol concentration, the enzyme was able to preserve 55% of its initial activity. At 30% ethanol concentration, its relative activity was 45% (Fig 4B). The results obtained in this study were interestingly very similar to the behaviour of GH1 family β-glucosidase, which was isolated from a hot spring metagenome and identified as BglM, against alcohol (Kaushal *et al*., 2021). The strong alcohol-tolerant ability of oregano β-glucosidase will allow it to be used efficiently in animal feed preparation processes.

CONCLUSION

In this study, β-glucosidase of *Origanum onites* L. used as a feed additive was purified for the first time and some biochemical properties were determined. It showed biochemical properties similar to exogenous enzymes recommended as enzyme additives. In particular, the enzyme's tolerance to glucose and alcohol meets the essential criteria expected from exogenous enzymes. Plants considered exceptionally safe are a good source of feed additives, as they can be applied directly without quantitative restrictions. Today, different aromatic plants are used as feed additives and their beneficial effects on animal health and nutritional quality of animal origin are being investigated. This study highlights the natural feed additive potential of *Origanum onites* L. In addition, this study may stimulate research on the increase in the nutritional value of *Origanum onites* L. when used as a feed additive.

Conflict of interest: None.

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