TURKISH JOURNAL OF SCIENCE
VOLUME IV, ISSUE I, 15-21
ISSN: 2587-0971

http://dergipark.gov.tr/tjos

Sığır Sütündeki Laktoperoksidaz Enzimi Üzerine Bazı Flavonoid Türevlerinin İnhibitör Etkisinin Belirlenmesi

Determination of Some Flavonoid Derivatives Inhibitory Effect on Bovine Milk Lactoperoxidase Enzyme

Aykut ÖZTEKİN[1,*]

[1] *Agri Ibrahim Cecen University, Vocational School of Health Services, Department of Medical Services and Techniques, Agri, Turkey*
**Sorumlu yazar / Corresponding Author*: aoztekin@agri.edu.tr

Geliş Tarihi / Received Date: 13 February 2019
Kabul Tarihi / Accepted Date: 30 March 2019

Öz: Peroksidazlar (PODs), bakteri, mantar, bitki ve hayvanlarda yaygın olarak bulunan, gıda, ilaç endüstrisi ve klinik teşhislerde önemli kullanım alanına sahip olan enzim gruplarıdır. Peroksidaz enzim sınıfına dahil olan Laktoperoksidaz (LPO) enzimi ise memelilerde süt, tükürük ve gözyaşında bulunur. Tiyosiyanat ve hidrojen peroksitle beraber enfeksiyonlara karşı vücudun savunma sistemlerinden birini oluşturur. Flavonoid türevleri bitkilerde bol miktarda bulunur ve diyetin önemli bir parçasını oluşturur. Sentetik olarak üretilen veya doğal olarak bulunan bu türevler, birçok farmakolojik aktiviteye sahiptir. Bu çalışmada laktoperoksidaz enzimi üzerine bazı flavonoid türevlerinin (5,7-dihidroksi-2-(3-hidroksi-4-metoksifenil)-4H-kromen-4-on (a), 3,5,7-Trihidroksi-2-(3,4,5-trihidroksifenil)-4H-kromen-4-on (b), 7-hidroksi-4'-nitroizoflavon (c), 6-Floroflavon (d), 7-Hidroksi-3-(4-metoksifenil)-4H-kromen-4-on (e), 7-metoksi-2-fenil-4H-kromen-4-on (f)) etkisi incelenmiştir. İlk olarak Sefaroz-4B-L-tirozin-sülfanilamid afinite kromatografisi ile sığır sütünden LPO enzimi 65 kat ve %23 verim ile saflaştırılmış ve bu enzim kullanılarak flavonoid türevleri ile kinetik çalışmalar yapılmıştır. 6 molekülün Ki değerlerinin 7.85 µM ile 0.023 µM arasında değiştiği bulunmuştur. 6-Floroflavon, 0.023 µM Ki değeri ile en etkili inhibitördür.

Anahtar Kelimeler — Laktoperoksidaz, flavonoid türevleri, inhibisyon.

Abstract: Peroxidases (PODs) are a group of enzymes that are commonly found in bacteria, fungi, plants and animals and have important uses in the food, pharmaceutical industry and clinical diagnostics. Lactoperoxidase (LPO) enzyme, which is included in peroxidase enzyme class, is found milk, saliva and tears in mammals. With thiocyanate and hydrogen peroxide, it forms one of the body's defense systems against infections. Flavonoid derivatives are abundant in plants and constitute an important part of the diet. These derivatives, which are produced synthetically or naturally, have many pharmacological activities. In this study, the effect of some flavonoid derivatives (5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one (a), 3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one (b), 7-Hydroxy-4'-nitroisoflavone (c), 6-Fluoroflavone (d), 7-Hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one (e), 7-Methoxy-2-phenyl-4H-chromen-4-one (f)) on the lactoperoxidase enzyme was investigated. Firstly, by using Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography, the LPO enzyme was purified 65 fold (with a yield of 23%) from bovine milk and kinetic studies were carried out with flavonoid derivatives using this enzyme. The Ki values of six molecules were found in ranging from 7.85 µM to 0.023 µM. 6-Fluoroflavone was the most effective inhibitor with Ki value of 0.023 µM.

Keywords — Lactoperoxidase, flavonoid derivatives, inhibition.

INTRODUCTION

Peroxidases are heme containing enzyme groups that found in bacteria, fungi, plants and animals [1]. LPO is a glycoprotein that is included in the peroxidase family and is found in saliva, milk and tears. LPO catalyzes the oxidation of thiocyanate to cyanide in the presence of hydrogen peroxide and shows antimicrobial effect [2]. The reactive products produced in the LPO/SCN^-/H_20_2 system oxidize sulfhydryl groups (- SH) of proteins in the bacterial cell membrane and inhibit the transport of nutrients, DNA and RNA synthesis and respiratory chain [3].

Flavonoids are a class of plant-derived polyphenolic compounds used in human health, pharmaceutical and industrial research [4, 5]. These compounds are predominantly found in vegetables, fruits and teas. These derivatives are formed by binding different groups to the two phenyl rings and a heterocyclic ring. There are more than 4000 types of flavonoid and are divided into six main groups according to their chemical structure: Flavones, flavonols, flavanols, flavanones, anthocyanidins and isoflavonoids [6, 7]. Flavonoids are an important part of our diet, especially in Japan, daily intake is up to 68.2 mg. Quercetin, one of the most known flavonoids, is abundant in apple and onion and contributes significantly to the daily intake of flavonoids [8, 9].

Studies have shown that flavonoids have many pharmacological effects, including antioxidant, anticancer and anti-inflammatory properties [10, 11]. These molecules have been shown to inhibit many metabolic enzymes such as monooxygenase, glutathione S-transferase, mitochondrial succinate-oxidase, nicotinamide adenine dinucleotide hydrate-oxidase, tyrosine and serine-threonine protein kinase [12].

In this study, it was aimed to investigate the inhibition effect of flavonoid derivatives with antioxidant activity on LPO enzyme. This study will define the interaction of these substances with the lactoperoxidase enzyme. For this purpose, the bovine milk LPO enzyme was purified and the inhibition effect of six flavonoid derivatives on the enzyme was investigated in detail.

MATERIAL AND METHOD

Chemicals and materials

The bovine milk used in the study was obtained from the local market. Amberlit CG-50-NH_4^+, CNBr-activated Sepharose 4B, L-tyrosine and sulphanilamide were purchased from Sigma-Aldrich and 5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one, 3,5,7-

Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one, 7-Hydroxy-4'-nitroisoflavone, 6-Fluoroflavone, 7-Hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one, 7-Methoxy-2-phenyl-4H-chromen-4-one were purchased from Fluorochem UK. All other chemicals used in this study were analytical grade.

Determination of LPO Enzyme Activity

Lactoperoxidase enzyme activity was calculated by absorbance increase at 412 nm of the coloured compound formed by the oxidation of the 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) chromogenic substrate in the presence of hydrogen peroxide (H_2O_2) [13].

Purification of LPO Enzyme

For the purification of LPO enzyme, bovine milk was first centrifuged at 3000g for 10 min and the milk fat was removed, then Amberlit CG-50-NH_4^+ was treated with fat-removed milk and washed with the appropriate solution to obtain a homogenate which, amounts of proteins were determined and enzyme activities were measured. The homogenate was applied to the Sepharose 4B-L-tyrosine-sulphanamide affinity column which equilibrated with 10mM phosphate buffer (pH 6.8). The affinity gel was washed with 25mM phosphate buffer (pH 6.8). The bovine LPO enzyme was eluted with same buffer (1 M NaCl/25, pH 6.8) [14]. Protein concentrations in the homogenates and purification steps were determined by the modified Lowry method [15].

Inhibition studies

The inhibition effects of flavonoid derivatives on LPO enzyme purified from Sepharose 4b-L-Tyrosine sulphanilamide affinity gel were investigated. Activity values for each derivative were measured at constant substrate (ABTS) concentration and at five different inhibitor concentrations. Then, activity %-inhibitor graphs were drawn and IC_{50} values were calculated from these graphs. Three different inhibitors and five different substrate concentrations were used for determination of K_i values. K_i and the inhibition type were calculated from Lineweaver–Burk graphics (1/V-1/[S])[16].

RESULTS AND DISCUSSION

Flavonoids are mostly phenolic compounds, abundant in fruits, vegetables and tea and are an important part of the human diet. Synthetic or naturally occurring flavonoid derivatives have many pharmacological activities. These are antitumor, anticonvulsant, vasorelaxant,

analgesic, antioxidant and anti-inflammatory activities [17]. It is known that natural and synthetic flavonoid derivatives are potent antioxidants as well as their regulatory effects on metabolism.

The effects of flavonoid derivatives on enzymes have been studied by many researchers and these studies are increasing day by day. In the literature, there are studies on the inhibitory effects of these derivatives on ACE (Angiotensin Converting Enzyme), Monoamine oxidase, lipase, acetylcholine esterase enzyme. Exemplarily, quercetin, quercetin-3-glucoside, quercetin-3-galactoside, cyanidin-3-galactoside were studied on the ACE and IC_{50} values were found to be 151 μM, 71 μM, 180 μM, 206 μM, respectively [18]. The effect of flavonoid derivatives on MAO-A (Monoamine oxidase-A) and MAO-B enzymes was investigated and it was observed that these derivatives showed an inhibition effect at μM level [19]. Luteolin and chrysoeriol have a noncompetitive and mixed inhibition on lipase enzyme with values of IC_{50}: 63 and 158 μM respectively [20]. In addition, synthetic flavonoid derivatives have been identified as potential inhibitors of AChE (Acetylcholinesterase) [21].

In this study, the effect of commercially available flavonoid derivatives on bovine milk LPO enzyme was investigated. First, the bovine milk LPO enzyme was purified using Sepharose 4B-L-tyrosine-sulphanilamide affinity gel. The results of purification are shown in Table 1.

Table 1. The Purification results of LPO enzyme from bovine milk.

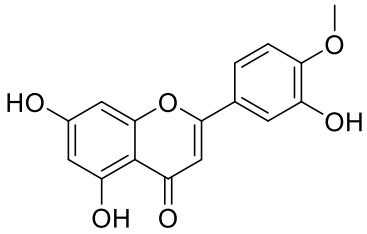
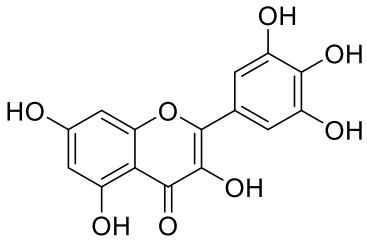
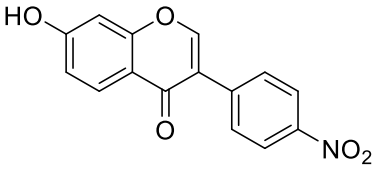
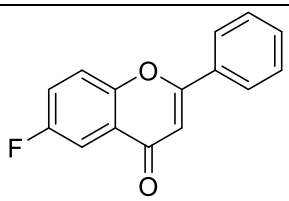
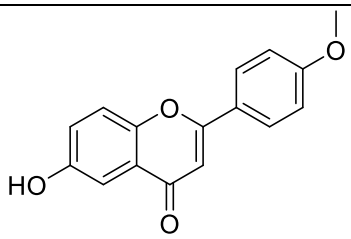
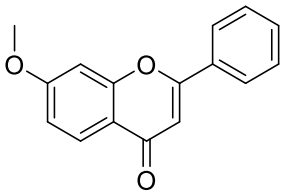
STEP	Total Volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total Activity (EU)	Total Protein (mg)	Specific activity (EU/mg)	Yield %	Fold
[a]***Step1***	6	0,9	8,2	5,4	49,2	0,109	100	1
[b]***Step2***	2	0,62	0,087	1,24	0,174	7,12	23	65

[a]*Step1: Amberlite CG-50-NH_4^+*

[b]*Step2: Sepharose 4B-L-tyrosine- sulphanilamide*

To determine the effect of flavonoid derivatives on purified LPO enzyme, detailed kinetic studies were performed and IC_{50}, K_i values and inhibition types were determined. The kinetics results are shown in Table 2.

Table 2. IC_{50} value, K_i constant and inhibition type of flavonoid derivatives for LPO enzyme

Inhibitors	**Name**	**IC_{50} (µM)**	**K_i (µM)**	**Inhibition type**
	5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one **(a)**	0,921	0,512 ± 0,0231	Competitive
	3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one **(b)**	0,434	0,452 ± 0,0918	Competitive
	7-Hydroxy-4'-nitroisoflavone **(c)**	0,832	0,623 ± 0,123	Competitive
	6-Fluoroflavone **(d)**	0,035	0,0233 ± 0,0081	Non-competitive
	7-Hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one **(e)**	1,81	2,86 ± 0, 261	Non-competitive
	7-Methoxy-2-phenyl-4H-chromen-4-one **(f)**	5,92	7,85 ± 0,712	Non-competitive

When the molecules and inhibition values are examined, the most effective inhibitor is 6-Fluoroflavone (d). The electronegative fluorine atom of the molecule **d**, increased the interaction of the inhibitor with the LPO enzyme and caused a stronger inhibition (See molecule **d,** K_i: 0.0233 μM). Table 2 shows that the inhibition effects of the molecules increase with the effect of OH groups. When the number of OH groups increases, the inhibition effect of the molecules is strengthened. Among the molecules containing the OH group, the highest effect was observed in molecule **b**. (K_i: 0.434 μM). This molecule contains six OH groups. The weakest inhibition value is K_i: 7.85 and molecule **f**. This is because molecule f does not contain the OH group and the electronegative group. It was observed that various groups that bind to flavon ring changed the inhibition effect to 350 fold.

In the literature, the inhibitory effect of nine different phenolic compounds on LPO enzyme was investigated and the K_i value of quarcetin was calculated as 5.99 μM. In the same study, K_i values of caffeic acid, ellagic acid, ferulic acid and syringic acid ranged from 2.83 to 17.76 μM [22]. In our study with flavonoid derivatives, the inhibition values ranged between 0.023 and 7.85 μM. This indicates that these derivatives, other than the molecule **f**, are more effective inhibitors.

CONCLUSIONS

Reducing the effect of LPO by the flavonoid derivatives, which is the most important part of the LPO/SCN^-/H_20_2 system, has negative consequences. The defense mechanism against bacteria is attenuated by the decrease in the activity of this enzyme. This makes especially babies less resistant to bacterial infections. In this study, six flavonoid derivatives were identified as the new reversible inhibitors of LPO enzyme and kinetic studies were performed. Ki, IC_{50} and inhibition types were determined for the first time.

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