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Antioxidative effect of Maillard reaction products of spermine–sugar system on partially purified plum polyphenol oxidase

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ABSTRACT

One economic issue that arises from the procedures used to produce dyes from naturally occurring phenolic chemicals that enzymes have oxidized is called "enzymatic browning". Polyphenol oxidase's existence in most fruits and vegetables is in charge of this problem. Adding antioxidants is one of the most crucial strategies for halting this process. We used spectrophotometry to examine the antioxidant effects of Maillard reaction products on this enzyme. Five hours of heating at 100 °C was used to study the properties and antioxidant activity of model systems including spermine and monosaccharides (glucose, fructose, and ribose). Vitamin C was used as a control to compare the DPPH radical scavenging activity of the MRP spermine-sugar model systems. Intermediate products at A_{294} and a browning intensity at A_{420} nm were increased with heating time. This increase was associated with a decrease in reduced sugar and free amino group contents. Polyphenol oxidase from Mirabelle plum (*Prunus domestica subsp. syriaca*) was fractionated by ammonium sulfate precipitation, dialysis, and ion exchange chromatography. A single peak was obtained with specific activity 11292.6 U/mg protein. The MRPs exhibited inhibitory effects on purified Mirabelle plum PPO from (42.6- 70.2%) compared with vitamin C which was 57.9%. Furthermore, the Lineweaver-Burk plots discovered the inhibition modes noncompetitive by the strongest system, spermine-glucose.

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1- Introduction

Commonly, plum fruit (*Prunus domestica*) is a stone fruit that belongs to the family Rosaceae of the genus *Prunus.* They have a variety of sizes, colors, tastes, and nutritional value[1, 2]. Due to their essential nutritional value, plums are a valuable food in our diet. Plumps are a rich source of the health-promoting compounds carotenoids, anthocyanins, and phenolic acids. Plums also have a high quantity of pectin, organic acids, sugars, tannins, aromatic compounds, and enzymes, making them low in calories and comparatively rich in nutritional value[3, 4]. Plums are available in different cultivars: Damson, Prune, Pershore, Greengage, Myrobalan, Victoria, and Mirabelle[5]. The Mirabelle plum (*Prunus domestica subsp. syriaca*) is thought to have been domesticated from a wild Anatolian fruit. It can be recognized by its oval shape, flesh with a smooth texture, and most importantly, its flecked yellow color (Figure 1). It is renowned for being flavorful and sweet. Although this fruit is usually used to make juice, it is also frequently fermented to make wine or distilled to create plum brandy. They are also widely utilized in the production of ja[6-8].

Figure 1: Mirabelle plum Fruit

The principal result of the Maillard reaction (MR), commonly referred to as "nonenzymatic browning," is Amadori chemicals, which are produced when carbohydrates react with protein N-terminal amino groups[9]. The reaction depends on temperature, pH, and reactant's kind[10]. This is typically documented that the Maillard reaction products (MRPs) affect the antioxidant capacity of foods[11, 12]. Antioxidant properties of MRPs are assumed to differ broadly and are powerfully influenced by the circumstances of the reaction and the procedures used for evaluation[13, 14]. They are frequently created in food products during thermal treatment or storage and utilized as inhibitors directly[15].

In numerous consumable plant products, polyphenol oxidase (PPO) catalyzes the oxidation of phenolic compounds in the attendance of oxygen throughout post-harvest handling and processing. Enzymatic browning products are dark-colored pigments that are produced by the fast

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condensing and polymerization of O-quinones, the principal oxidation products that are produced. Due to the degradation of the product's nutritional, functional, and organoleptic qualities, brown pigmentation is typically regarded as harmful to food quality[16]. Several studies have indicated that polyphenol oxidase is inhibited by MRPs, for example, MRP inhibited PPO isolated from potatoes and apples[17] mushrooms, and eggplant[18]. However, the inhibitory effects varied depending on the enzyme origin and nature of the reactant type of MRP[19]. The study aimed to examine the ability of MRPs to inhibit partially purified PPO activity from mirabelle plum and prevent enzymatic browning.

Experimental part

Materials:

Spermine, gallic acid, and vitamin C were obtained from Sigma-Aldrich Co. Catechol (o-dihydroxybenzene) and polyethylene glycol are purchased from BDH Co. Glucose, Fructose, and Ribose from Fluka Co.

2-Methods

Preparation of MRPs:

Spermine-sugar model systems were produced according to [20]. Each system was created by mixing 0.05 M ribose, fructose, and glucose individually with 1% spermine. After that, each mixture was transferred to screw-sealed tubes, tightly capped, and heated in a water bath at 100 C. After heating for 0, 1, 2, 3, 4, and 5 hours, the samples were taken. The heated samples were properly immersed in ice water to cool. Each MRP sample was kept at 4°C until it was analyzed and used.

Antioxidant activity measurement using the DPPH radical scavenging technique

Three milliliters of DPPH solution (5 mg/10 mL) were combined with two milliliters of MRP solution, and 25 milliliters of methanol were added. The mix was vigorously shaken before being left in the dark for 45 minutes. At 515 nm, the decrease in absorbance was monitored. The results were expressed as milligram gallic acid equivalents per gram of MRPs[21]. Vitamin C was used as a control antioxidant.

Analyses

Colorless intermediate and browning intensity measurement:

Spectrophotometrically, a colorless intermediate was detected at A_{294} nm and the intensity of the brown color was estimated by following A_{420} nm[22].

Reducing sugar measurement:

To determine the reducing sugar concentration, 1 ml of the samples and 1 ml of dinitrosalicylic acid were added to a test tube. The mixture was heated for five minutes at 100°C. Then cool at room temperature and add 8 ml of distilled water. At 540 nm, absorbance has been determined. Using glucose (10–100 mg/ml), a standard curve was created[23].

Free amino group measurement:

To determine free amino acids, 0.2 ml of ninhydrin reagent is added to 1 ml of sample. The mixture was heated at 100 °C for 10 minutes and then cooled at room temperature. Absorbance was measured at 570 nm. The standard curve was done using lysine (5-50 mg/ml)[24].

Reducing power measurement:

Within a glass tube, 1 ml $K_4[Fe(CN)_6]$ (1%) was added together with 1 mL of the sample and 0.5 mL sodium phosphate buffer (0.2 M,

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pH 6.6). Following a 20-minute incubation period at 50 °C, 1 milliliter of 10% TCA had been added to the mixture. The mixture was subsequently centrifuged for 10 minutes at 805 xg. Once 1 ml of supernatant was obtained, 200 µL of 0.1% FeCl3 and 1 ml of D.W. were added to it. The absorbance was estimated at 700 nm. Absorbance increase served as the indicator of reducing power[25].

Determination of protein content:

Total protein conc. was estimated, using BSA as a standard at 650 nm[26].

Enzyme assay:

Using catechol as a substrate, PPO activity was measured spectrophotometrically. One milliliter of 0.01 M catechol, 0.9 ml buffer (pH 7.2), and 0.1 ml of purified enzyme were mixed. The absorbance was determined at 420 nm with a 10-second interval for 5 minutes after the enzyme was added. One unit of PPO was used as the enzyme quantity, resulting in a 0.001 unit increase in absorbance per minute[27].

Enzyme extraction:

Fresh mirabelle plums purchased from a local market were used to extract PPO. It was cleaned and peeled. In the presence of 2% PEG, 40 grams was homogenized with 100 ml of phosphate buffer (0.1M, pH 7.2) using a blender for 2 min. Enzyme-containing juice was centrifuged for 20 minutes at 10000 xg after being filtered. The resulting extract was subjected to 80% of $(NH_4)_2SO_4$ precipitation. Then the pellet was dissolved in a small volume of the same buffer[28].

Dialysis:

Dialysis was carried out by placing an enzyme-containing solution in a dialysis tube against phosphate buffer (0.1 M, pH 7.2). The solution was stirred at 4˚C with six-time changes of buffer.

Ion exchange chromatography:

The dialyzed solution was placed on a column containing a CM-cellulose cation exchanger. Phosphate buffer was used to elute a protein at a flow rate of 0.7ml/min (each fraction 3 ml). The protein was followed using absorbance at 280nm. The fractions containing PPO activity were detected, pooled, and lyophilized then used in the following experiments[29].

Determination of PPO molecular mass

The PPO molecular mass was estimated by using slap-electrophoresis which was applied to the concentrated protein extract obtained from the former separation step. Sodium dodecyl sulfate gel electrophoresis was used. The protein bands were visible when applying the Coomassie Blue staining method[24]. A standard curve was adopted to evaluate the molecular mass of PPO.

Enzyme inhibition:

The inhibitory effect of PPO was studied by adding 0.2 ml MRPs to the reaction mixture of the PPO assay that measured as described above from a range of 0-5 hours.

Inhibition mode of PPO:

The inhibition of purified PPO was studied by using the spermine-glucose system as an inhibitor. The activity was followed at 420 nm using (0.25-3mM) of substrate after

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incubation of 0.2 ml enzyme with 0.1 ml of inhibitor for 30 minutes[30].

3-Results and Discussion

MRPs with varying browning and color appearances are anticipated to form in each system, aiding in comprehending their free radical scavenging properties. The system is permitted to react with DPPH to evaluate the produced MRPs' ability to scavenge free radicals. One type of chromogenic radical that can interact with antioxidants directly is DPPH. DPPH, a stabilized radical, is frequently employed to assess primary antioxidant activity[31, 32]. Table 1 demonstrates the scavenging influence of MRPs on DPPH radicals. All MRPs were discovered to have antioxidant properties. Among them, the system glucose- spermine, showed higher scavenging effects (0.49 mg. gm^{-1}) besides vitamin C.

Table 1: The scavenging effect of MRP (mg gm^{-1}) to quench DPPH radicals

MRP product	($mg.gm^{-1}$)
Glucose- spermine	0.49
Fructose- spermine	0.45
Ribose- spermine	0.34
Vitamin C	0.61

MRPs have excellent antioxidant properties in many food products due to metal ion chelation, radical chain and H_2O_2 breakdown, and reactive oxygen species scavenging[33]. MRPs produced by chitosan-sugar systems showed increased DPPH radical scavenging activity. The most effective ones were those derived from chitosan-glucose[34]. The MR generated by an amino-sugar system was linked to the generation of compounds with high antioxidant activity. The MRPs produced from glucose and amino acids (lysine, histidine, and methionine) revealed significant scavenging properties in addition to vitamin C and catechin[18].

On the other hand, spermine is one of the polyamine compounds, which is characterized by its small molecular weight and alkali behavior because it contains four amine groups[35]. It is synthesized from L-ornithine or by the decarboxylation of amino acids. This compound has a scavenging ability against various ROS. Its antioxidant properties depend mainly on chelating minerals[36-38].

Variations in UV (A_{294}) and browning intensity (A_{420})

According to Ajandouz et al., absorbance at A294 nm and A420 nm indicates the presence of colorless intermediate molecules and ending browning compounds[39]. A slow variation in A_{294} nm of entirely MRPs derived from spermine-sugar model systems was initiated with an increasing heating interval of up to 2 hr. Subsequently, a severe increase, specifically with glucose was observed as the heating period enhanced up to 5 hr. as illustrated in Figure 1.

This rise coincided with the findings of Phistut and Jiraporn (2013), who reported that MRPs produced from glucose and chitosan exhibited the greatest increase in A_{294} nm, followed by those derived from lactose, fructose, and maltose, respectively[34]. A rise of A_{294} nm was observed by Lerici et al. (1990) when the glucose-glycine combination was heated. According to Benjakul et al. (2005), the heating period of the porcine plasma protein

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reducing sugar model system was found to cause a constant increase in A_{294} nm[40].

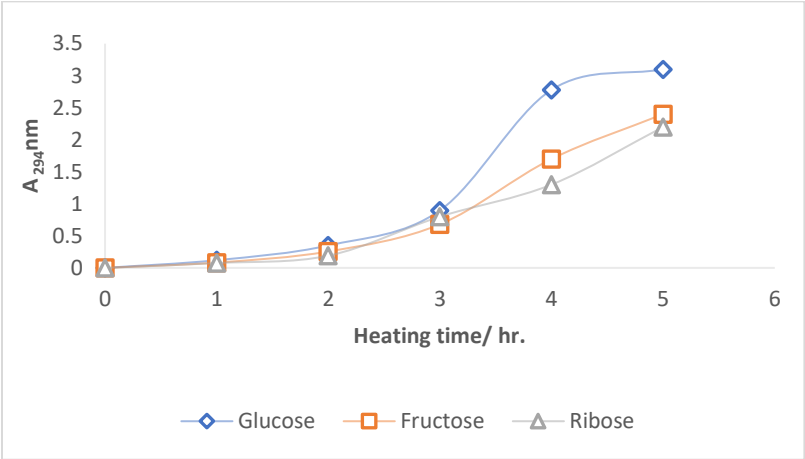

Figure 2: Variations in A_{294}nm with different heating periods

The easiest observable consequence of the MR is brown color development (A_{420} nm), which can be assumed visually. Its intensity indicates the advanced state of MR and frequently serves the extent to which MR is present in meals. As proved by the increase in browning intensity through heating, some intermediate products appear to be converted to the final brown compounds, while others appear to be generated by both reactants. Browning intensity at A_{420} of all MRPs gradually increased to 5 hr. of heating. The highest absorption was clear for the Spermine-glucose model system compared to other sugars (Figure 3).

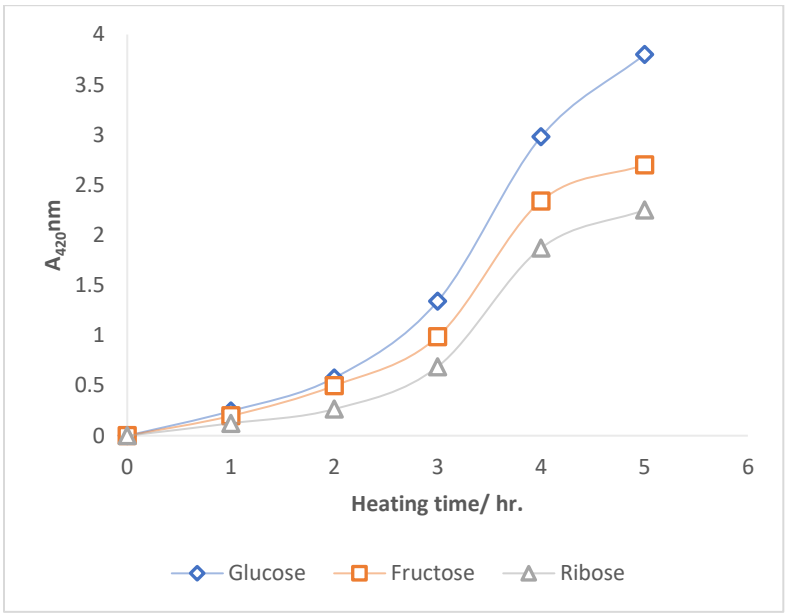

Figure 3: Variations in A_{420}nm with different heating periods

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For MRPs derived from Chitosan-Glucose, fructose, and lactose, A_{420} nm increased distinctly after heating[34]. Conversely, some researchers discovered that fructose contributes more to browning than glucose[41]. This indicated that the formation of brown pigments was directly proportional to the creation of intermediate products.

Variations in free amino group content

All MRPs developed from spermine-sugar systems have their reactive amino groups modified, as seen in Figure 4. The amount of amino groups in all MRP samples decreased with increasing heating time, and it increased when spermine and glucose reacted. According to this discovery, spermine's amino group is covalently linked with sugar to generate a more glycated product, especially as the heating time increases.

Phisut and Jiraporn (2013) revealed that the free amino groups' chitosan declined little by little as a result of the MR with monosaccharides glucose, lactose, and fructose respectively [34]. During continued heating of a casein–sugar system, lysine decline was demonstrated as mentioned by [42].

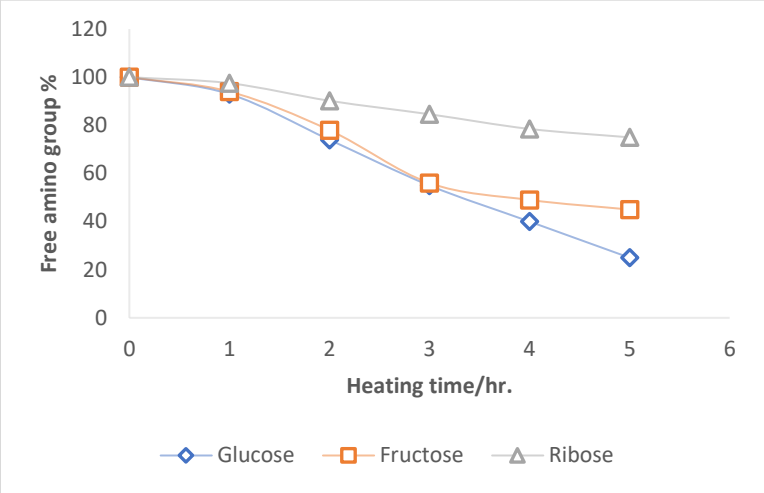

Figure 4: Variations in free amino group content with different heating periods

Variations in reducing sugar content

Figure 5 illustrates a progressive decline in the reduced sugar content of all MRPs made from spermine-sugar solutions. The MRPs obtained from the spermine-glucose model system demonstrated a dramatic drop in decreased sugar content. This outcome demonstrated the use of glucose as a reactant in MR, demonstrating a quicker rate of reaction than other sugars.

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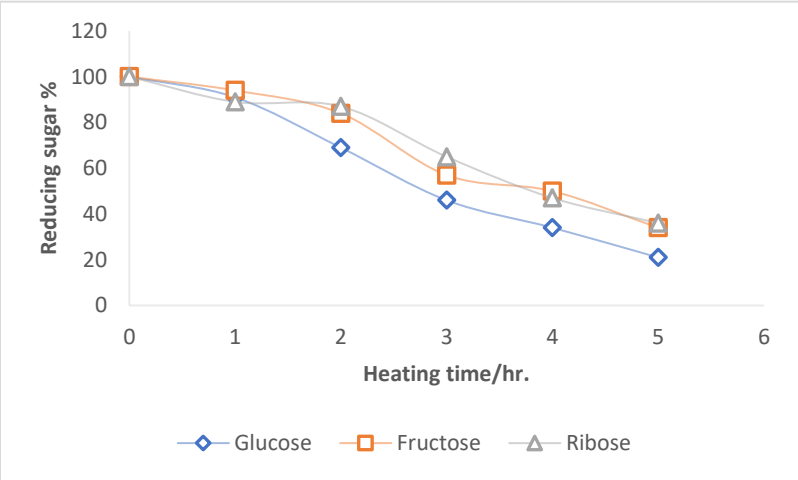

Figure 5: Variations in reducing sugar with different heating periods

When heat-induced MR is applied to chitosan-glucose, casein-glucose, and fructose-lysine, the reduced sugar content decreases[20, 34]. According to the findings, the decrease in the free amino group and reduced sugar content corresponded to an increase in browning intensity A_{420} and A_{294}. This revealed that prolonged heating catalyzed the interface of amino groups in spermine with reducing sugar through glycation development. As a result, glucose was more reactive than other sugars in the formation of glycated spermine, as evidenced by the greatest decrease in free amino groups with a concurrent increase in browning. The reaction rate of glycation may depend on the acyclic formula and the carbonyl groups' electrophilicity[42].

Enzyme Purification:

When sedimentation with 80% $(NH_4)_2SO_4$, the sp. activity of the enzyme has given rise to 234.79 U/mg protein with a purification fold of 0.54 related to the crude. These results indicate that Mirabelle plum PPO was magnificently purified to 26.31 fold. As can be seen, the protein content diminished afterward-successive purification steps but the specific activity value increased from 343.28 to 11292.6 U/mg protein (Table 2). The elution profile of PPO by column chromatography exposed a lone peak (Figure 6).

Table (2): Purification steps of PPO from Mirabelle plum (*Prunus domestica subsp. syriaca*)

Purification steps	Volume (ml)	Total protein (mg)	Total activity U*	Sp. Ac. (U/mg protein)	Yield %	Purification Fold
Crude extract	94.4	27.74	11903.64	343.28	100	1
Ammonium sulfate	16.8	18.09	5311.09	234.79	35.69	0.54
Dialysis	12	6.41	3371.37	420.73	22.65	0.97

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CM-Cellulose	34.5	0.192	2721.52	11292.6	18.28	26.31

* A unit is defined as the amount of enzyme that oxidizes one micromole of substrate per minute at 25 °C.

PPO purification from Sapodilla plums (*Achras sapota*) illustrates increased activity after precipitating by 80% of ammonium sulfate with a specific activity of 762.7 UE. One peak of the enzyme was separated by using sephacrylS-200 chromatography molecular mass was approximately 66 kDa[43]. PPO was purified from Damson plum using ammonium sulfate precipitation, dialysis, and finally, affinity purification by Sepharose 4B-L-Tyrosine-p-aminobenzoic acid with purification-fold 93.88[44]. The enzyme was extracted from Stanley plums (*Prunus domestica L.*) with 36-fold via $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose and Sephadex G-100[45]. On the other hand, this enzyme was purified from different sources including, Hemsin Apple[46], peaches[47], apricot[48] by using various techniques.

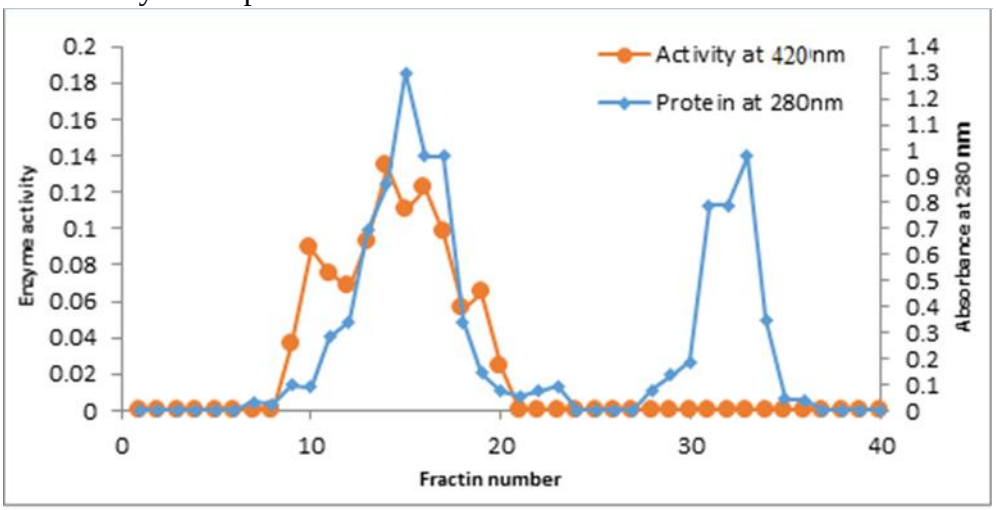

Figure 6: Elution profile of Mirabelle plum PPO by CM-cellulose column (2×25 cm)

Molecular weight:

A single clear band of enzyme was revealed after applying the SDS-PAGE electrophoresis technique. The protein band migrated, close to a molecular mass of pepsin (24 kDa). The molecular mass of purified Mirabelle plum PPO was approximately calculated to be 22 kDa (Figure 7). Also, by using SDS-PAGE gel electrophoresis, Das et al., (1997) found a single polypeptide band with 25 kDa of pineapple's PPO. A single protein band of purified PPO from sapodilla plum and damson plum was estimated to be about 29 and 50 kDa respectively [43]. On the other hand, different molecular masses of PPO have been described before such as in banana 41 kDa [49], and atemoya fruit 82 kDa [50].

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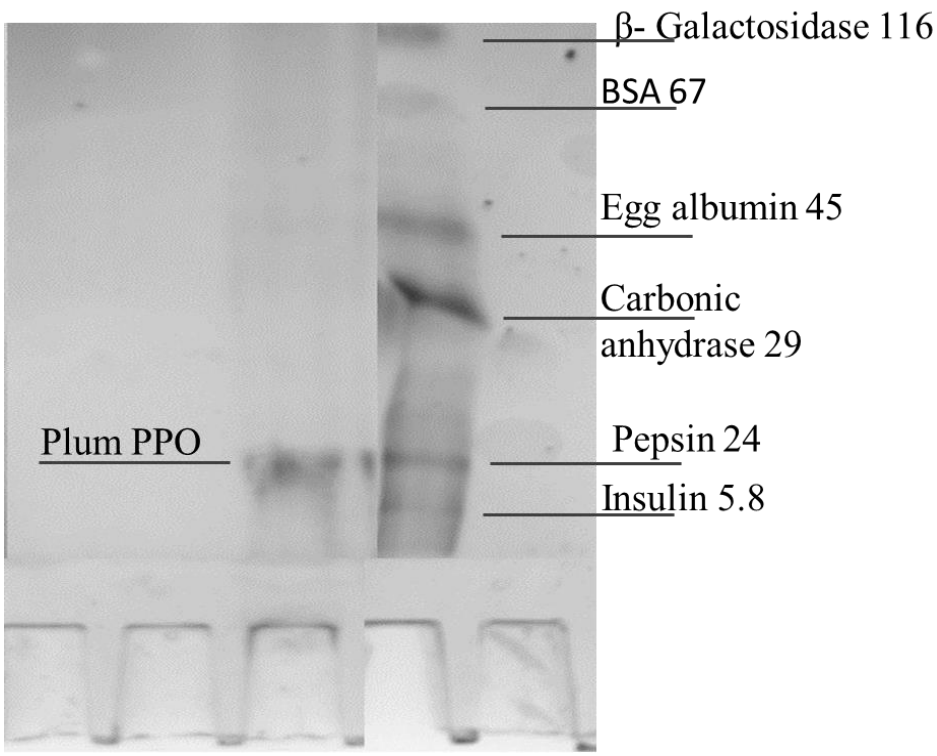

Figure 7: SDS-PAGE electrophoresis of PPO purified from Mirabelle plum with standard proteins.

PPO inhibition:

MRPs, such as Amadori rearrangement products, have the potential to scavenge oxygen radicals and chelate metals. Another distinguishing feature is the PPO inhibitory effect, which may help to prevent enzymatic browning in vegetables and fruits[51]. It was observed that MRPs inhibited the purified PPO from plum. The impact of diverse MRPs of sugars and spermine on enzyme activity is conveyed in Figure 8.

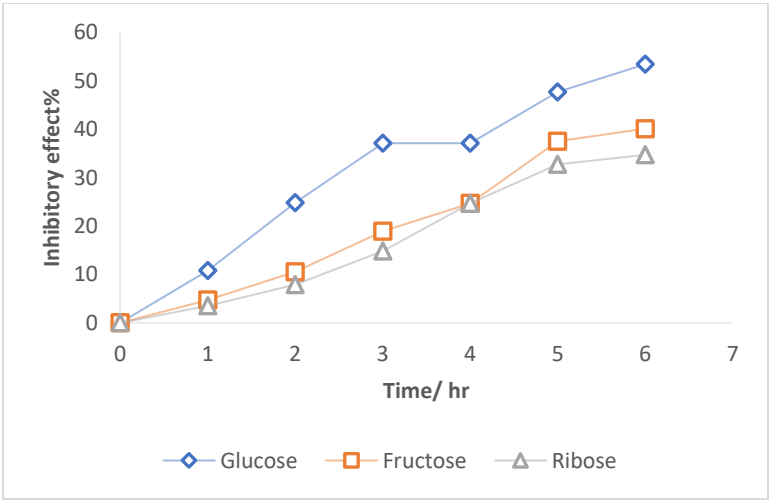

Figure 8: Inhibitory effect of spermine-sugar system on purified PPO from Mirabelle plum

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Many studies have shown that MRPs are capable of inhibiting the PPO activity in foods. Billaud et al. (2005) investigated the MRP effect of glutathione or cysteine with different monosaccharides and disaccharides as antibrowning agents on PPO activity in mushrooms, apples, and eggplant[17]. On the other hand, the MRPs synthesized from glucose with tyrosine, proline, and glumatic acid individually, exposed inhibitory effects on enzymes isolated from potatoes and apples[18]. The presence of MRPs caused the noodles to be significantly less dark in color, which was attributed to MRPs' inhibitory effect on PPO[52]. Further, Figure 9 shows an evaluation of MRP and vitamin C inhibitory effects on PPO compared to the control.

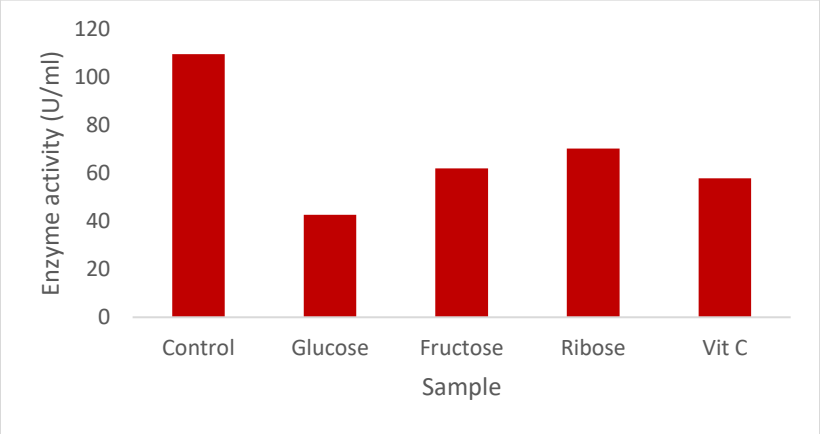

Figure 9: Comparison between the inhibitory effects of MRPs solutions and vitamin C) on PPO extracted from Mirabelle plum

It was shown that spermine–glucose had extra inhibitory effects than other MRPs and vitamin C, however, the inhibition of rest MRPs was significant (42.6- 70.2%). According to the literature, MRPs derived from glucose with glycine initiated a significant decline in enzymatic browning in apples and mushrooms[53]. Certain fractions generated during the MR have been shown to have the ability to either inactivate enzyme activity or form PPO inhibitors. These compounds' ability to suppress oxidation processes is based on several mechanisms, including reducing activity, free radical scavenging, and metal-ion chelating[54].

Inhibition mode of PPO

The inhibition of the purified plum PPO activity was investigated with the existence of a spermine-glucose system. The inhibition mode was verified by drawing a Lineweaver-Burk plot (Figure 10) using different concentrations of catechol as substrate. The results exhibited a non-competitive inhibition mode. The value of Vmax was reduced from 0.212 units /ml/ min) without inhibitor to 0.101 units/ml/ min) in the presence of inhibitor, while the Km value remained approximately constant at 2.56 mM. This enzyme has been non-competitively inhibited in previous studies after being purified from different plant sources such as quince[55], purslane[56], and potato[57].

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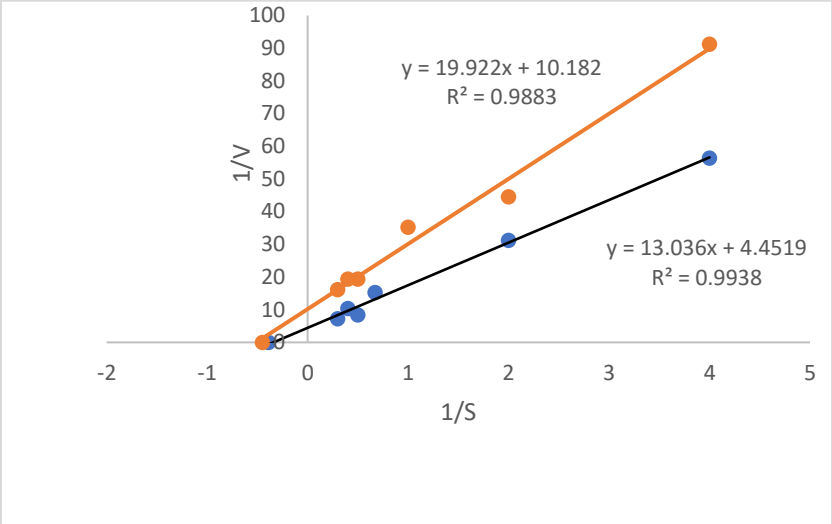

Figure 10: The inhibition mode of purified plum PPO by the spermine-glucose system

4-Conclusion

Enzymatic browning is an economic problem that happens as a result of processes complicated in the production of dyes from enzymatically oxidized phenolic compounds of natural origin. PPO present in fruits and vegetables, is responsible for this phenomenon. One of the most important methods used to stop this reaction is adding antioxidants**.** The antioxidant properties of MRPs are recognized in the formation of reductone structures that have both reducing and metal complexing properties. Numerous model systems were used for this purpose. Additionally, MRPs were revealed to have powerful radical scavenging activity, which was significant when compared to the known antioxidant vitamin C. MRPs from spermine-sugar systems exhibit high antioxidant properties, with glucose being the most effective reactant for MR with spermine's amino groups, resulting in highest browning pigment formation. The strongest inhibitory effect of MRPs on PPO extracted from Mirabelle plum was found with the spermine-glucose system. We conclude that enzyme inhibition by compounds may be due to their antioxidant capacity or chelation with the copper ion, a cofactor of PPO.

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