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Potential Application of Casein for the Inhibition of β-Glucuronidase Activity

Dilek Demirbüker Kavak[1], Bilge Akdeniz[2],

[1,2]*Afyon Kocatepe University, Engineering Faculty,Food Engineering Department, Afyonkarahisar.*

e-mail: dkavak@aku.edu.tr

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Abstract

β-glucuronidase enzyme (EC. 3.2.1.31) is a hydrolytic enzyme and has an important role in glucuronidation mechanism. Changes in this enzyme concentration can lead development of adverse metabolic changes in the human body leading certain diseases and therefore inhibition researches for this enzyme are in the scope of different biomedical and nutritional applications. The aim of this study is to evaluate the inhibitory potential of casein towards β-glucuronidase (GUS) enzyme and characterization of the inhibition of GUS enzyme by casein compound. Inhibition kinetics was carried out in a dose dependent way under simulated physiological conditions. GUS enzyme inhibition assay and measurements of kinetic parameters were used to analyze the inhibitory effect. Results showed that casein had high antioxidant activity and its activity depended on its concentration under simulated physiological conditions. Kinetic studies indicated that casein had a potential to be used as glucuronidase inhibitor and showed a mixed type inhibition mechanism towards the GUS enzyme.

β-GlukuronidazAktivitesinin İnhibisyonunda Kazeinin Kullanım Potansiyeli

Anahtar Kelimeler
İnhibisyon, kinetik, kazein, β-glukuronidaz

Özet

β-glukuronidaz enzimi (EC. 3.2.1.31) hidrolitik bir enzimdir ve glukuronidasyondaönemli bir role sahiptir. Bu enzim konsantrasyonunda meydana gelen değişiklikler insan vücudunda olumsuz metabolik değişikliklere yol açacak hastalıklara neden olabilmektedirr. Bu sebeple bu enzimin aktivitesinin inhibisyonu ile ilgili olarak biyomedikal ve beslenme alanlarında çeşitliçalışmalar söz konusudur. Bu çalışmanın amacı, kazeinin β-glukuronidaz (GUS) enziminin inhibisyonu amacıyla kullanım potansiyelinin araştırılması ve kazein ile GUS enzimi arasındaki inhibisyon mekanizmasının tanımlanmasıdır. İnhibisyon kinetiği çalışmaları yapay fizyolojik koşullar altında, doza bağlı şekilde gerçekleştirilmiştir. GUS enzim inhibisyon deneyi ve kinetik parametrelerin ölçümleri, enzimin inhibe edici etkisinin analiz edilmesi amacıyla kullanılmıştır. Sonuçlar, kazeininyapay fizyolojik koşullarda yüksek antioksidan aktiviteye sahip olduğunu ve bu antioksidan aktivitenin konsantrasyona bağlı olduğunu göstermiştir. Kinetik çalışmalar, kazeinin glukuronidaz inhibitörü olarak kullanılabilecek potansiyele sahip olduğunu ve GUS enzimi içinkarışık tip inhibisyon mekanizması sergilediğini göstermiştir.

1. Intorduction

β-glucuronidase enzyme (EC. 3.2.1.31) is one of the most important hydrolytic enzymes in living systems (Ünak et al. 2005). Enzyme liberates the toxins and mutagens that have been glucuronated in the liver and excreted into the gut with the bile (Dabek et al. 2008). Bacterial β-glucuronidases present in the intestine (Cardona et al. 2006). Its activity is responsible for hydrolysis of xenobiotic glucuronide conjugates in the gut, and can thus produce toxic aglycones and carcinogenic substances (Kavak, 2010). This hydrolysis may liberate free toxins and mutagens which can lead to high local concentrations of carcinogenic compounds where colon mucosa exposed (Fujisawa et al.2001; Rafter et al. 2004; Cardonaet al. 2006).

Casein is one of two major protein fractions in milk. It is about 40% of the protein in human milk and between approximately 78% and 86% of the protein in cow, sheep, goat milks. In milk nearly 80-95% of the casein is in the form of colloidally dispersed particles, known as micelles (Phadungath, 2005). As the casein is one of the major proteins in milk, with its high percentage; it plays a fundamental role in human nutrition. It has very important role to be a source of amino acids required by growth of the neonate. Besides being a nutritional source for young mammals and also it is source of a number of bioactive peptides with immunostimulating, antimicrobial, antioxidant activities (Field et al. 2008; Almajano et al. 2007, Silva et al. 2005, Swanson et al. 2002).

Casein has been the subject of increasingly intense studies according to its biological activities in health area. Since casein has high bioactivity, it is important to investigate the interaction of casein with GUS enzyme, responsible for hydrolization of toxic substances in gut. Therefore aim of this study is to investigate the possible interaction of casein with *E.coli* originated GUS enzyme under simulated physiological conditions. Within this scope, antioxidant activity of casein will be investigated and possible kinetic inhibitory mechanism will be revealed.

2. Materials and Methods

2.1. Materials

β-glucuronidase (*E.coli* origin), glysine, phenolphthalein glucuronide were used in enzymatic assays and were obtained from Sigma Chemical Company (Germany). Reagents used for the antioxidant activity assay; ABTS (2,2-Azino-bis-(3-Ethylbenz-Thiazoline-6-Sulfonic acid) is obtained from the same chemical company. Casein is obtained from Merck (Germany). All the other reagents used in this study were of analytical grade.

2.2. Preparation of Simulated Physiological Conditions (SIF)

Experiments were performed in water bath using a solution that simulates physiological conditions (SIF) which was phosphate buffer at pH 6.8 (75 mM). This solution met optimum working conditions for GUS activity (recommended by the purchaser instructions) where the shaking rate and the temperature were also set to 100 rpm and 37^{o}C, respectively.

2.3. *Antioxidant Activity Assay*

ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation decolourisation assay was used for the assessment of antioxidant activity of casein samples according to procedure given by Demirbüker et al. (2004). Scavenging ability of samples for ABTS radical cation (ABTS+*) at different concentrations were measured using UV-visible spectrophotometer 734 nm. Sample without casein was used as blank.

2.4. *β-Glucuronidase Activity Assay*

Enzyme activity assays were performed in SIF solution (pH 6.8; 75 mM phosphate buffer, 37^{o}C). Spectrofotometric activity measurements were at 540 nm performed using “Fishman Method” for 30 min. incubation time (Fishman and Bernfeld, 1955). Phenolphthalein glucuronide solution was used as substrate and GUS enzyme activity results was defined as unit where one unit will liberate 1.0 µg of phenolphthalein from phenolphthalein glucuronide per hour at pH 6.8 at 37°C.

2.5. *Kinetic Analyzes*

To understand the inhibition of a GUS enzyme by casein as inhibitor; kinetic analyses were performed. Casein concentrations used in this study were 0.125 and 0.18 mg/mL. Kinetic analyzes were performed using Michaelis Menten Kinetics where the velocity of the reaction (V) was the µg phenolphthalein liberated from phenolphthalein

glucuronide for one hour per mg enzyme.

3. Results and Discussion

3.1. Antioxidant Activity of Casein in SIF

Antioxidant activity results for casein were tabulated in Table 1. Results showed that casein had antioxidant activity in SIF and this activity was increased with the increase in casein concentration. Results were in good agreement with the previous literature studies. It was reported that different casein fractions such as β-casein and α-casein showed radical-scavenging activities in aqueous solution (Almajano et al. 2007).

Table 1. Radical inhibitory effect of casein at different concentrations

Casein Concentration (mg/mL)	0.08	0.125	0.18	0.36	0.72
Radical Inhibition (%)	24.3 ±0.3*	26 ±1.1	29.7 ±0.7	31.3 ±0.9	34.3 ±1.4

**Standard deviations*

This high antioxidant activity might be due to the active protein residues in the complex casein structure. Main physiological role of casein in the milk system was widely accepted to be a source of amino acids required by growth. However, these proteins suggested to play another important role. Bzducha and Wolosiak (2006) suggested that antioxidant activity of casein may be due to capability of free radicals capturing (histidine residues) or reducing them on the way of making the hydrogen atom available e.g. tyrosine, phenylalanine. Since casein is one of the major protein in milk, it plays a fundamental role with its high antioxidant property in human nutrition.

3.2. Effect of Casein on GUS Activity

Enzyme GUS could be activated or inhibited with different doses of inhibitors. Therefore different doses of casein were used to investigate GUS enzyme activity with respect to different concentrations of the casein. Results of the inhibitory effect of casein on GUS were given in Table 2.

Table 2. Effect of casein on GUS activity at different concentrations

Casein Concentration (mg/mL)	0.08	0.125	0.18	0.36	0.72
Enzyme Activity (%)	90.2 ±1.4	84.4 ±1.1	76.3 ±0.5	70.7 ±1.4	65.3 ±0.9

Results showed that casein has a potential inhibitory effect on enzymatic activity. Inhibitory effect was increased with the increase in casein concentration. Highest inhibitory effect was obtained at 0.72 mg/mL casein concentration where residual enzyme concentration was 65.3 %. This inhibitory potential of casein was used in different inhibition experiments (Swanson et al. 2002; Wang et al. 2000) due to its micelluar structure. This structure possibly provides interacting and binding of the surrounding constituents.

3.3. Results of Inhibition Kinetics

For the kinetic studies, varying amounts of substrate were added to enzyme solution and the reaction velocity; V was measured for each substrate concentration [S]. The plot was given in Figure 1.

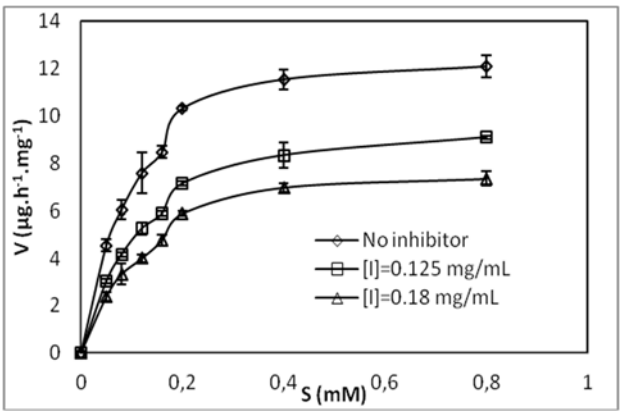

Figure.1. Effect of casein on GUS enzyme reaction rate ([I]: inhibitor-casein concentration).

The resulting curve takes the form of a hyperbola which the velocity values initially increase steeply but eventually approach a maximum level (Vmax). V reached "plateaus" indicated that V became independent of [S] at large values of [S].

To determine the kinetic parameters, Line-weaver Burk plots (double reciprocal plots) were plotted to apply Michaelis Menten kinetic approach. Michaelis-Menten equation is the rate equation for an enzyme– catalyzed reaction and is the mathematical description of the hyperbolic curve.

The formula is:

$$V_o = \frac{V_{max}[S]}{K_m[S]} \qquad (1)$$

where, Vo is the initial velocity, Vmax is the maximum velocity, [S] is the substrate concentration Km (Michaelis-Menten constant) is the substrate concentration at which the reaction velocity is the half of the maximum velocity.

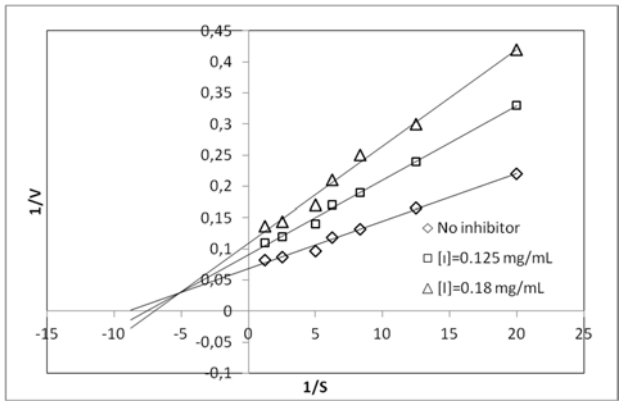

Figure 2. The double reciprocal plots for the enzyme (control) and enzyme inhibitor systems.

The double reciprocal plots for the enzyme (control) and enzyme inhibitor systems were drawn (Figure 2). Results showed mixed type inhibition mechanisms due to their intercept. In mixed inhibition, the inhibitor binds to a site of the enzyme other than the active site and elicits a conformational change. Simple kinetic parameters determined from the double reciprocal plots were given in Table 3. As a result, the apparent Vmax decreases and the apparent Km may increase or decrease which indicates the affinity of the enzyme towards substrate (Kavak et al. 2010).

Table 3. Apparent(*) kinetic parameters

Sample	Vmax*	Km*
No inhibitor	14.66	0.11
[I]=0.125 mg/mL	11.06	0.13
[I]=0.125 mg/mL	9.21	0.14

As mixed inhibitors it was found casein did not bind directly in the active site, and therefore did not block substrate binding (Segel, 1993; Marangoni, 2003). The interaction of inhibitors with GUS enzyme possibly distorted the active site of GUS enzyme to a non optimal conformation for catalysis which reduced the reaction velocity.

4. Conclusions

According to the results, it was found that casein had a high antioxidant capacity indicating a possible preventive role in cancer risks by eliminating the free radicals attack. Kinetic studies indicated an effective inhibition for the GUS enzyme. Thus, this effective inhibition might take a preventive role in human intestinal system caused by microbial flora. This antioxidant property together with the effective GUS inhibition makes casein a potent molecule for nutritional and biochemical applications in the perspective of reducing cancer risks for the future applications.

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