Effect of camel whey immunoglobulins concentrate on the activity of sheep liver glutathione-s-transferases

Abdallah Lubna*, Benali Sanad, Zugier Diaa, Hoge Diaa, Bradia Hassan, Nazzal Abdulla, Al-Aqtum Ahmad and Kabaha Mahmoud
Department of Biology and Biotechnology, Faculty of Science, An-Najah National University, Nablus, PALESTINE
*alubna@najah.edu

Abstract
The aim of this study was to find out the effect of camel whey immunoglobulins on the activity of glutathione-s-transferases (GST). The effect of camel whey immunoglobulins was tested at variable conditions including their concentration effect, their pre-incubation effect and the effect of pH and temperature on their activity. Moreover, the effect of camel whey immunoglobulins on the thermal and pH stability of GST was also examined. Furthermore, kinetic parameters of GST were studied in the presence of camel whey immunoglobulins.

The observed results showed that camel whey immunoglobulins were not affected by different pH values. Interestingly, milk immunoglobulins preserved the stability and the activity of GST enzyme except in the strong acidic and strong alkaline media. Camel whey immunoglobulins were more stable at 40 ºC and their effect on GST activity declined after 60 ºC. It was clearly noticed that the pre-incubation of GST enzymes with immunoglobulins for a certain period caused better effect than their direct addition. GSH Kinetic results showed that camel whey increased Vmax and Km values. In conclusion, camel whey immunoglobulins play an important role in the activation of GST enzymes even at different conditions.

Keywords: Camel, glutathione-s-transferase, immunoglobulins, whey.

Introduction

Glutathione-s-transferase isoenzymes (GSTs) are widely distributed in nature and they are present in different organisms such as microbes, plant, fish, insect, birds and mammals[1]. Glutathione-s-transferases contain two distinct super families of enzymes that possess transferase activity[2]. The first family is cytosolic, while the second one is membrane-associated protein in eicosanoid and glutathione metabolism (MAPEG)[3,4]. The cytosolic GSTs are involved in the metabolism of foreign chemicals such as carcinogens, environmental pollutants and cancer chemotherapeutic drugs as well as in the detoxification of harmful endogenously derived reactive compounds[1].

Most of these enzymes can form thioether bond between the sulfur atom of reduced glutathione (GSH) and substrate by catalyzing the conjugation of reduced glutathione with compound that contain an electrophilic center[3,5]. It was found that specific isoenzyme of GSTs were over expressed in a wide variety of tumors and may had a role in the etiology of other diseases, so it was regarded as a promising therapeutic target[6].

Camel milk was considered as the main food for humanity because it has essential nutrients[7]. Over and above, camel milk is better than other ruminant milk as it contains good qualities of lactoferrin, lysozyme, lacto-peroxidase and immunoglobulins[8]. Moreover, camel milk had many properties that makes it very useful choice to be used in some parts of the world to cure certain diseases. In this aspect, whey proteins in camel milk had an important role as an anti-tumor and anti-carcinogenic agent[9]. Furthermore, those proteins provided a substrate for GSH synthesis which caused an increase of GSH concentration in tissues[10]. Several studies on camel lactoferrin reported that it was a multifunctional protein as it had anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, antioxidant and anti-tumor properties[11,12].

Besides that, other studies showed that lacto-peroxidase was involved in the natural host defense system against invading microorganisms[13]. Likewise, immunoglobulins from camel milk are also very important therapeutic agent. This is due to the presence of heavy chain antibodies which are devoid of the light chains[14]. The small size of these immunoglobulins enabled them to be found in the camel milk[15]. Therefore, heavy chain antibodies were used in the immune therapy for various disorders such as cancer, multiple sclerosis and Alzheimer's disease[12,16].

In addition to that, camel's whey proteins were more heat resistant than cow whey proteins[8,17]. This could be referred due to the presence of heat stable proteins such as heavy chain antibodies. For antibodies, functional stability is critical, especially under the effect of temperature, pressure, chemicals and other factors. In contrast to conventional antibodies, camel heavy chain antibodies and their derived nanobodies showed great stability under different conditions[18,19].

From this point of view, this research was carried out to examine the effect of camel whey immunoglobulins concentrate on the activity of GSTs. Their effect was demonstrated at different conditions regarding their concentrations, medium pH and temperature.

**** Author for Correspondence***

Material and Methods

Preparation of whey immunoglobulins concentrate: Milk samples were collected from one female camel (Jenin, West Bank). For immunoglobulins concentrate preparation, the casein was precipitated from the pooled skimmed milk samples based on Brussow et al technique of milk renneting with commercially available rennin to obtain good crude contraction[20]. The total protein content of camel whey sample was determined by Biuret method[21].

Purification of sheep liver glutathione-s-transferases: Fresh sheep liver was homogenized at 4°C with 50 mM phosphate buffer containing 1 mM EDTA pH 7. The homogenate was centrifuged at 10,000 ´g for 25 min. The obtained supernatant was centrifuged at 50,000 for 120 min and the supernatant that contains cytosolic glutathione-s-transferases was precipitated by ammonium sulfate at 30-70%. The obtained solution was purified by gel filtration column chromatography using (Ultrogel ACA 44 column, Sigma) with 0.2 M phosphate buffer pH 7.

The protein level for all fractions was determined using Warburg and Christian method[22] and GSTs activity was determined by Habig et al method[23].

The fractions with GSTs activities were pooled and applied to affinity column (GSH-agarose, Sigma). The column was equilibrated and washed with 0.2 M phosphate buffer pH 7. After elution, all fractions were determined for GST activity and protein level[22,23]. The fractions with GSTs activities were pooled, lyophilized and subsequently used for the activity and kinetic and studies.

Determination of GST activity at various concentration of camel whey immunoglobulins concentrate: Whey immunoglobulins concentrate was prepared at different concentrations (2, 4, 6, 8 and 10 mg/ml) in addition to 0.0125, 0.025. 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml, then the effect of each concentration on the activity of GST was measured by a spectrophotometer at 340nm[23].

Determination of GST activity at different incubation times with camel whey immunoglobulins concentrate: Five mg/ml whey immunoglobulins concentrate was mixed with 10 µg/ml GST and incubated in ice at different time intervals (0, 10, 20 and 30 min). After each incubation period, GST activity was measured by a spectrophotometer at 340nm[23].

Stability studies

pH effect on the activity of camel whey immunoglobulins concentrate: For *in vitro* pH stability measurement, all camel whey immunoglobulins samples were incubated for 10 min at room temperature in phosphate buffered saline with different pH values (2, 4, 6, 7, 8, 10 and 12). Then GST activity assay was performed to determine their activity at each pH value in comparison to samples tested at all studied pH values without using whey immunoglobulins[23].

Thermal effect on the activity of camel whey immunoglobulins concentrate: *In vitro* thermal stability of camel whey immunoglobulins was done by their incubation at different temperatures (0, 10, 20, 30, 40, 50, 60, 70, 80, 90°C and 100°C) for 10 min each. Then GST activity assay was done to determine their activity at each temperature in comparison with normal sample[23].

Effect of camel whey immunoglobulins concentrate on pH stability of GST: To determine the effect of whey immunoglobulins on the pH stability of GST; 5 mg/ml whey immunoglobulins were mixed with 10 µg/ml GST and incubated at different pH values (2, 4, 6, 7, 8, 10 and 12) for 10 min. After incubation, all mixtures were tested for GST activity[23].

Effect of camel whey immunoglobulins concentrate on thermal stability of GST: To determine the effect of whey immunoglobulins on the thermal stability of GST, 5 mg/ml whey immunoglobulins were mixed with 10 µg/ml GST and incubated at different temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90°C and 100°C) for 10 min. After incubation, all mixtures were tested for GST activity[23].

Kinetic properties of purified glutathione-s-transferase enzymes: The kinetic values of GST for GSH were determined by the procedure as follows: different concentration of GSH (0.05, 0.15, 0.25, 0.35, 0.5, 0.75, 1, 1.25 and 1.5 mM) was varied. While the concentration of CDNB was fixed at 1.5 mM. 50 ul of purified enzyme (10 ug/ml) was added to 850 ul of the working reagent (reduced GSH) and 50 ul distilled water and the reaction was initiated by adding 50 ul of starting reagent (CDNB). Degree of absorbance at 340 nm was recorded for one min. Km and Vmax were calculated using Hanes plot and equation. The same procedure was used to determine the effect of whey immunoglobulins on GST Km and Vmax values except that 50 ul distilled water were replaced by 50 ul of 5 mg/ml whey immunoglobulins.

Results

Effect of camel whey immunoglobulins concentration on GST activity: After purification, affinity fraction was subjected to dialysis and lyophilized. The final obtained concentration from GSTs was 200 *u*g/ml. The specific activity for purified GSTs was approximately 84 *umole/min/mg.* It was observed that the gradient increase in the camel whey immunoglobulins concentrations leads to an increase in the activity of GST enzyme. Figure 1 and 2 showed that there was a slight increase in enzyme activity at low concentrations of whey immunoglobulins. At higher whey immunoglobulins concentrations, the rate of enzyme activity increased significantly as the best effect of camel whey immunoglobulins was at 8 and 10 mg/ml.

Determination of GST activity at different incubation times with camel whey immunoglobulins: Figure 3 showed that the direct addition of whey immunoglobulins to

GST increased the activity of the enzyme up to 35%. While the pre-incubation of GST enzyme with whey immunoglobulins for 10 minutes increased its activity up to 70%.

Stability studies: The obtained results showed that the activity of the enzyme alone increased gradually until it reached the neutral medium and then the enzyme activity decreased at higher pH values. In the presence of pre-incubated whey immunoglobulins at different pH values, the activity of GST was increased when compared with its activity without whey immunoglobulins (Figure 4).

Figure 5 showed that GST was inactive and unstable at very low and high pH values. The maximum values of GST activity were almost in the neutral medium. The results clearly showed that in the pH values confined between 4 and 10, whey immunoglobulins maintained enzyme activity and prevented the instability of GST enzymes at these pH values.

The results for the thermal treatment of whey immunoglobulins were shown in figure 6. There was a difference in GST activity in the absence and presence of whey immunoglobulins that were previously incubated at different temperatures. The effect of whey immunoglobulins on enzyme activity increased with raised temperature. The highest activity of whey immunoglobulins on GST was between 40°C and 60°C. At higher temperatures, the effect of whey immunoglobulins on GST activity began to decline at temperatures higher than 80°C.

The effect of camel whey immunoglobulins on GST activity at different temperatures was studied as shown in figure 7. The GST activity started to decline at 40°C and almost all activities were lost at 50°C and higher temperatures. In the presence of whey immunoglobulins, GST activity increased at temperatures between 10°C-50°C. On the contrary, at 60°C and higher temperatures whey immunoglobulins could not influence the activity of GST.

Kinetic properties of purified glutathione-s-transferase enzyme: The relationship between GSH and the activity of GSTs in the absence and presence of immunoglobulins was shown in figure 8. When the initial activity of GST is plotted versus substrate concentration, it was observed that the activity of the enzyme increased in a concentration dependent manner up to a rather high substrate concentration after which a plateau with saturation was attained. The initial activity of GST reaction was measured related to substrate concentration and plotted in accordance with the Hanes plot and equation analysis. The plot gives Km and Vmax values. Km value for GST increased from 0.025 mM to 0.031 mM after addition of whey immunoglobulins. For Vmax, it also increased from 16.1 µmol/min/ml to 18.2 µmol/min/ml in the presence of whey immunoglobulin.

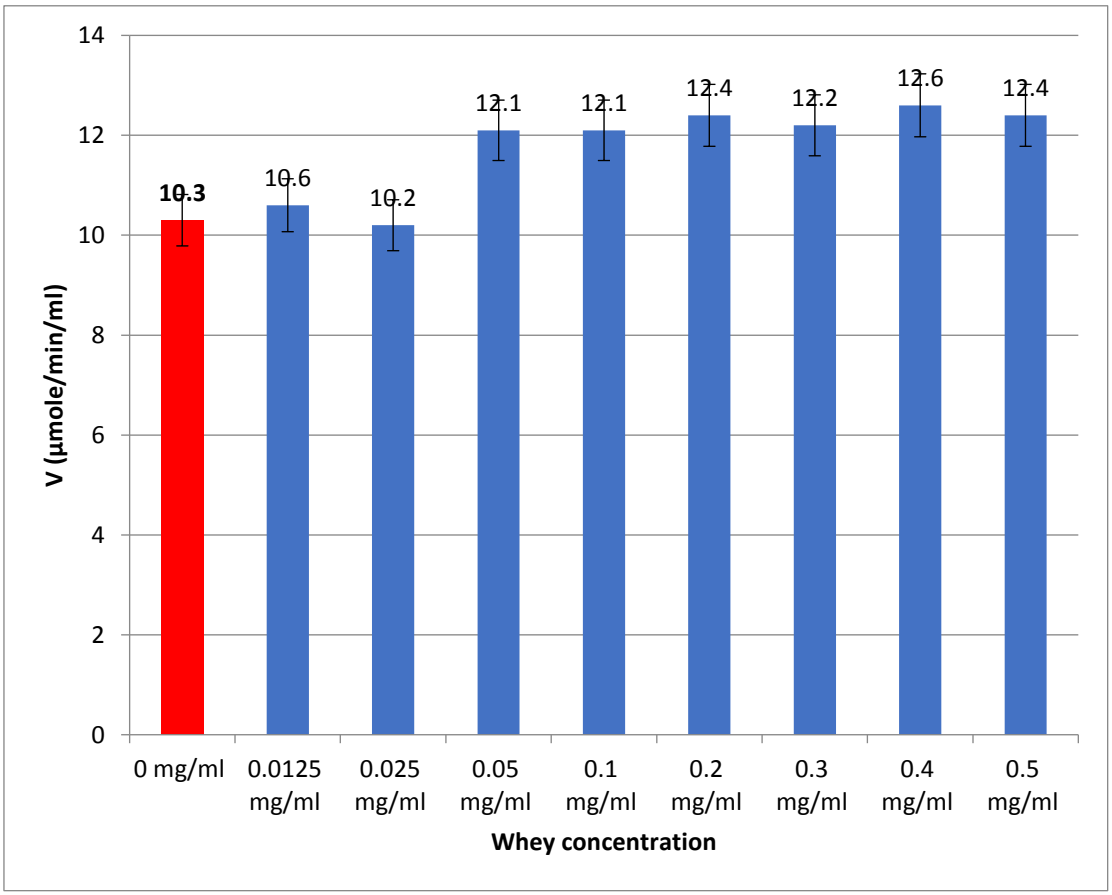

Fig. 1: Effect of whey immunoglobulins concentrate on GST at low concentrations

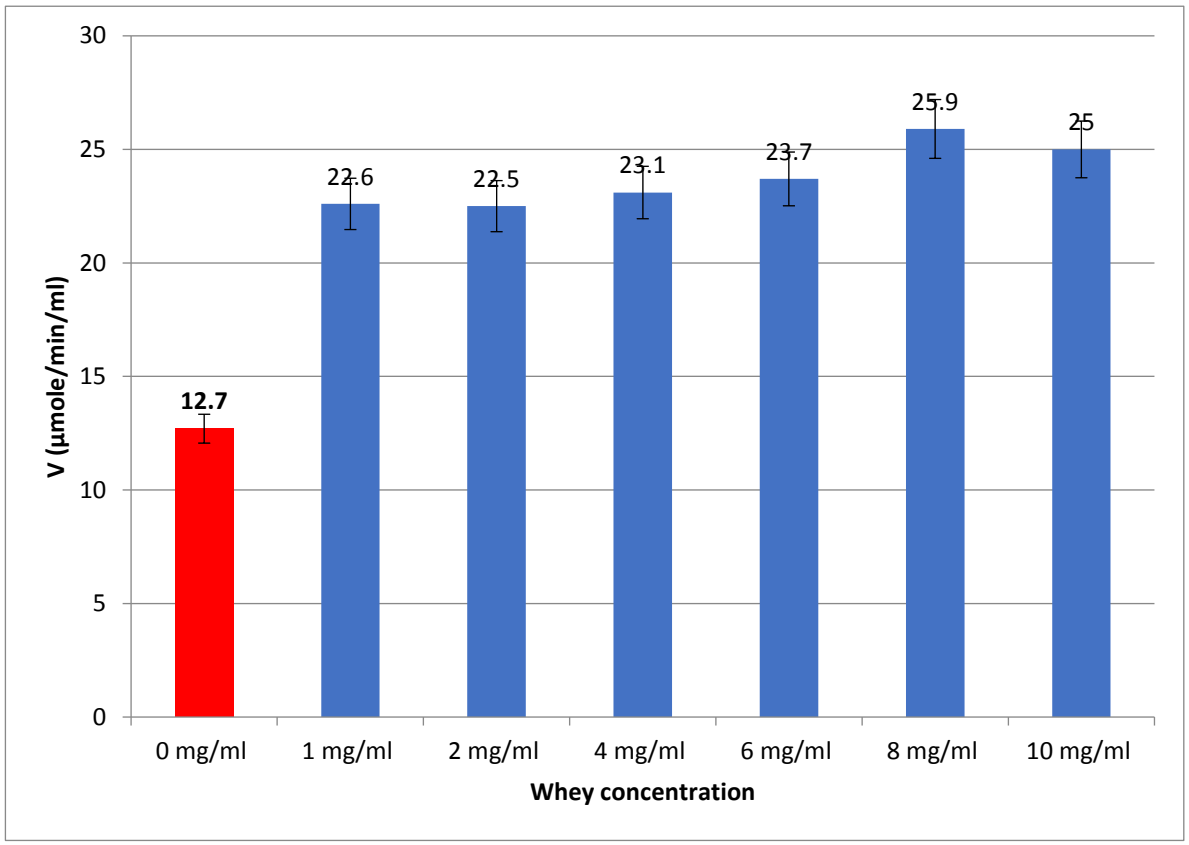

Fig. 2: Effect of whey immunoglobulins concentrate on GST at high concentrations

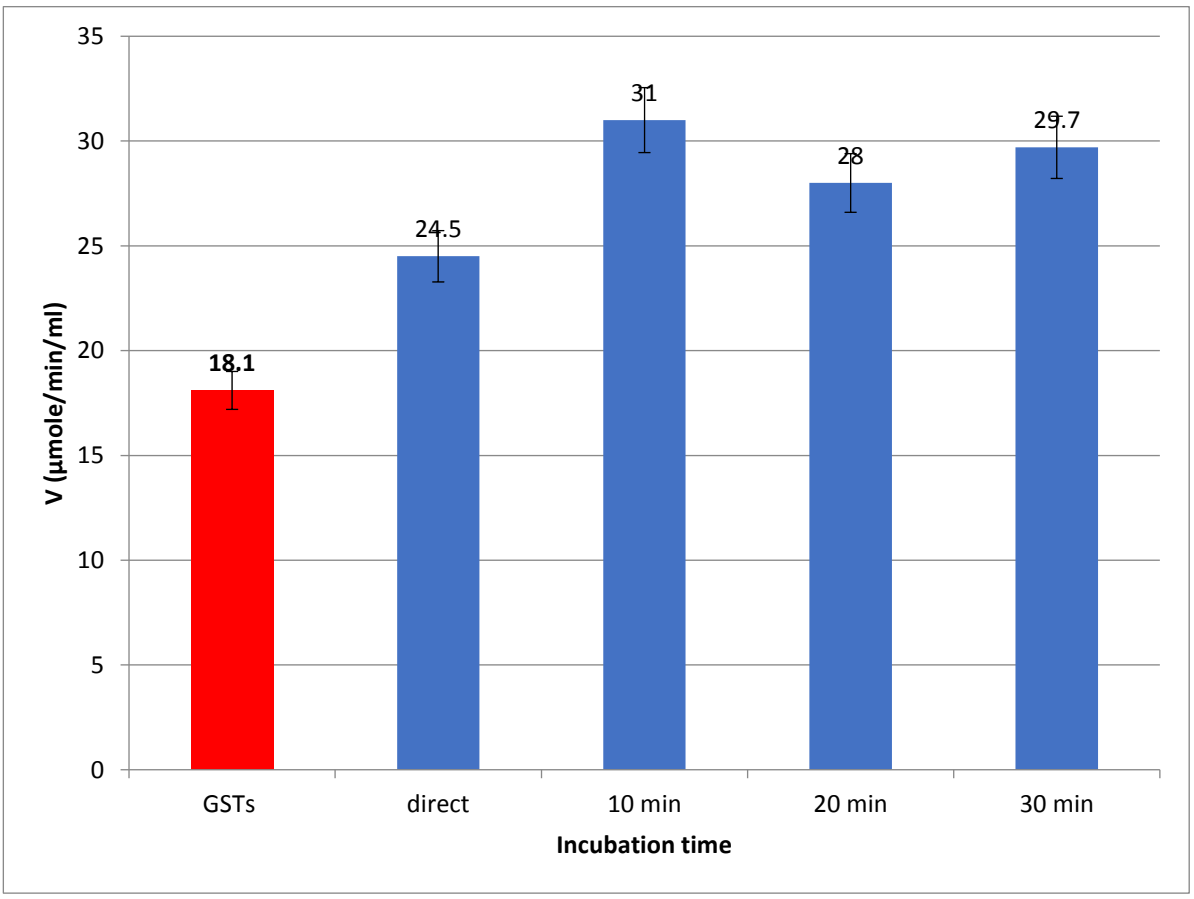

Fig. 3: Effect of whey immunoglobulins concentrate incubation time on GST activity.

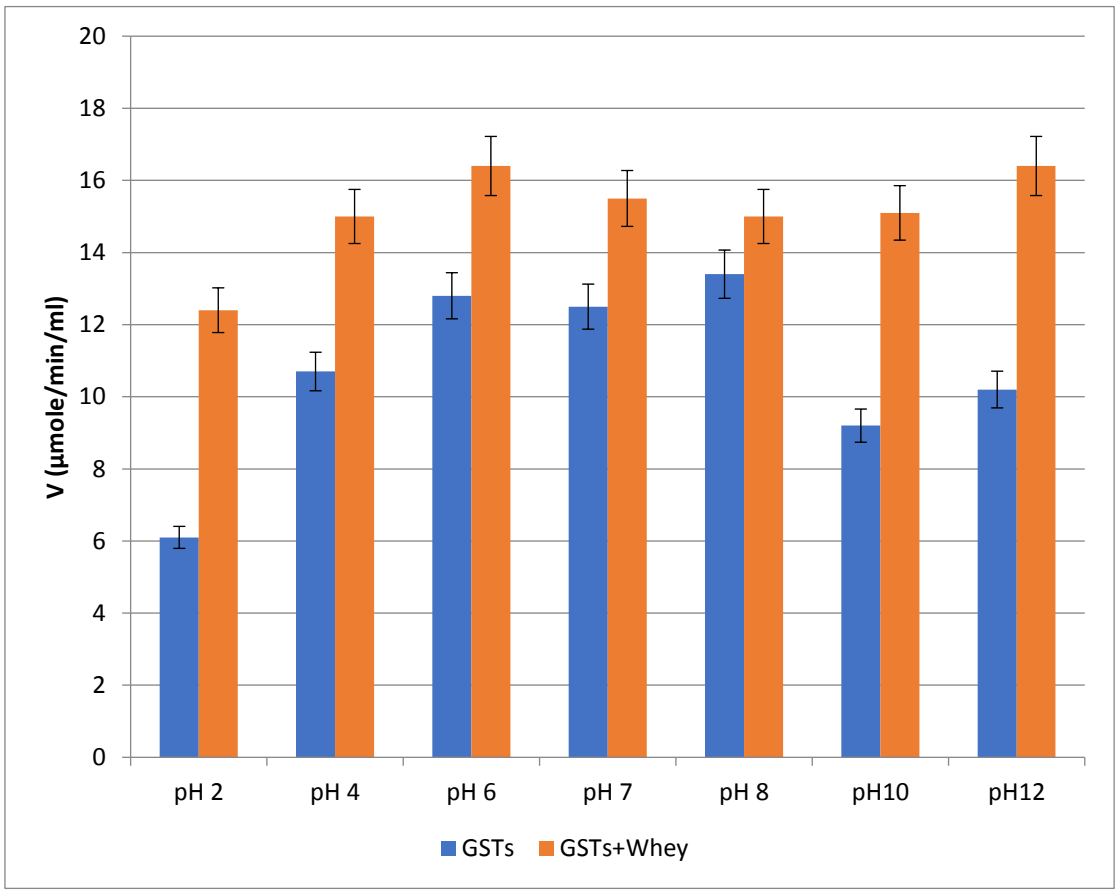

Fig. 4: pH stability of camel whey immunoglobulins concentrate.

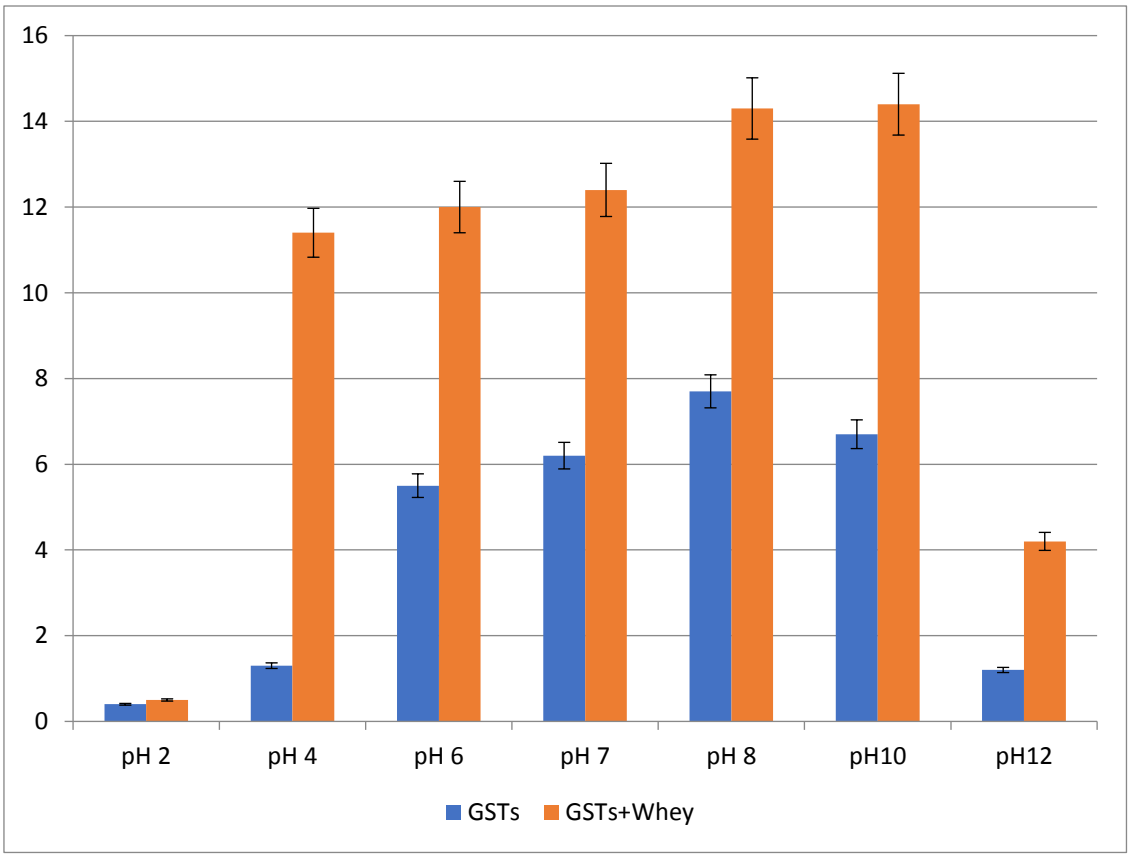

Fig. 5: Effect of camel whey immunoglobulins concentrate on the pH stability of GST.

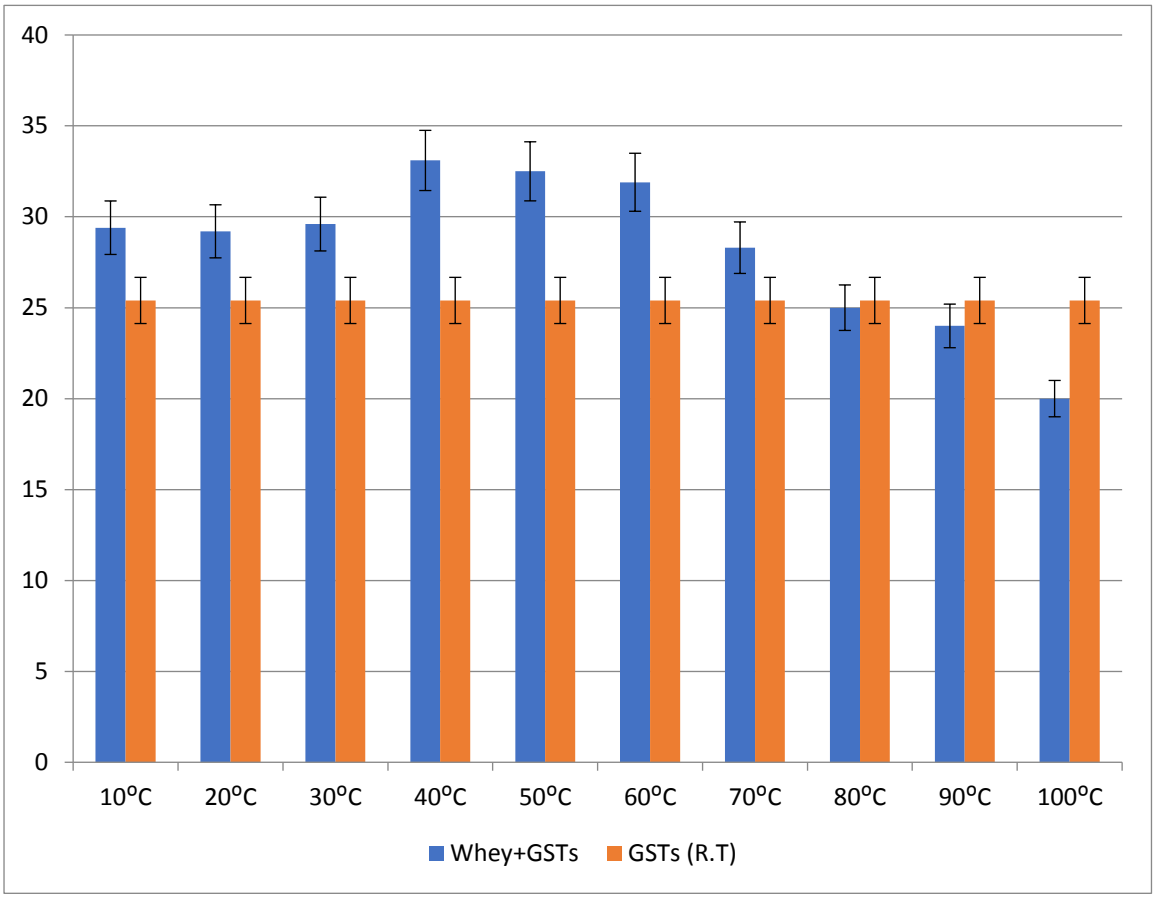

Fig. 6: Thermal stability of camel whey immunoglobulins concentrate.

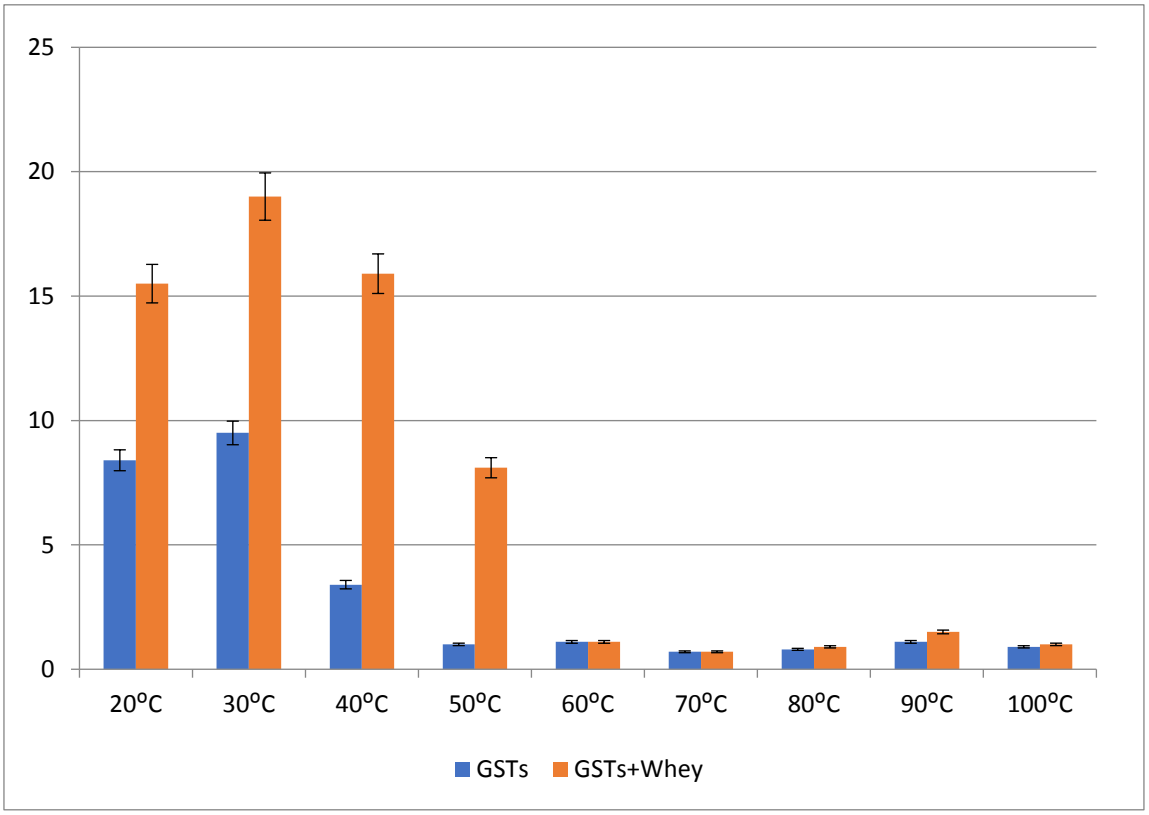

Fig. 7: Effect of whey immunoglobulins concentrate on GST thermal stability.

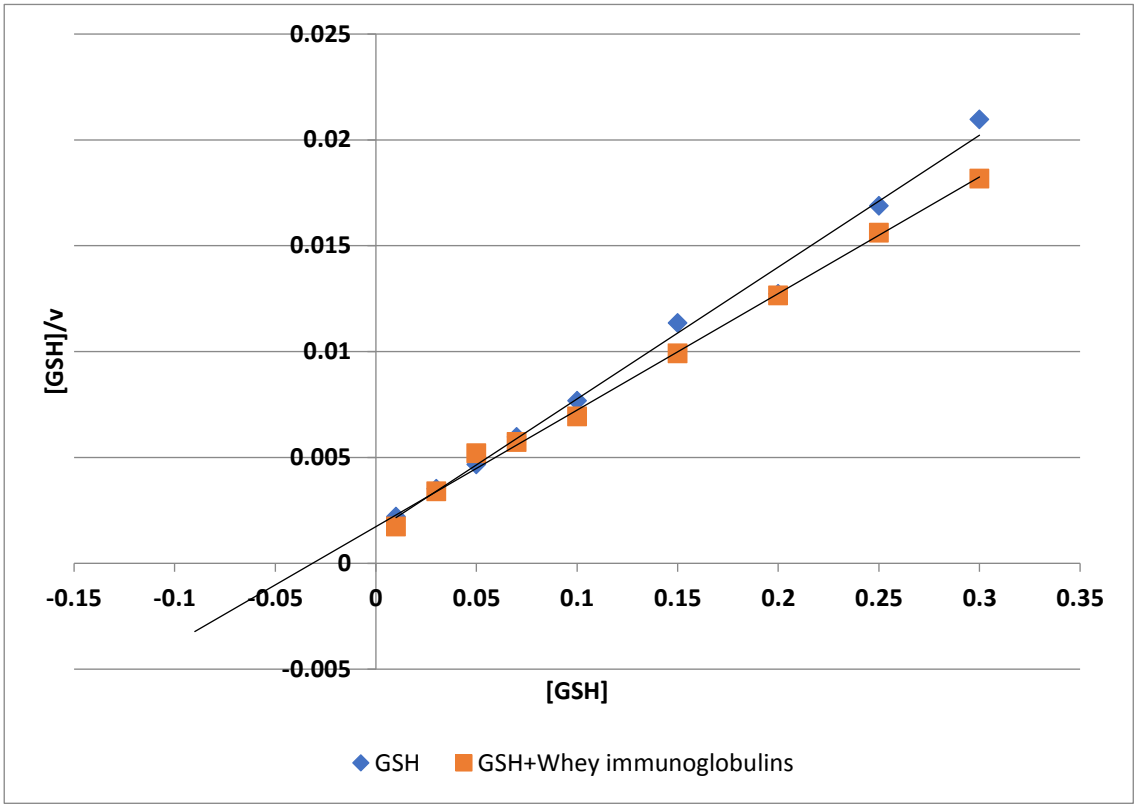

Fig. 8: Hanes-plot of GSTs activity versus GSH in the presence and absence of whey immunoglobulins concentrate.

Discussion

The purpose of this study was to find out and observe the camel whey immunoglobulins effect on the purified sheep liver GST activity. The crude extract of sheep liver was purified in three purification steps, then the pooled active fractions were assayed for GST activity using CDNB as substrate. The specific activity for purified GST found to be 84 µmol/min/mg. Obtained results were higher than the GST specific activity of sheep liver that purified by glutathione-affinity and wash-batch method[24]. The differences in GST specific activity within the same species in several studies may be due to number of factors including age, sex, diet and genetic polymorphisms. In addition to that GST activity may be altered by the exposures to drugs and environmental pollutants[25].

The effect of whey immunoglobulins on GST activity was measured at different conditions: whey immunoglobulins concentration, incubation periods, pH and temperature. Whey immunoglobulins concentrations affected GST activity positively in direct relationship. According to the obtained results, the most suitable whey protein concentration for better GST activity was 8 and 10 mg\ml. However, these results can be supported by other study[26], they found that treatment of wounded diabetic and non-diabetic rats with whey proteins leads to a significant increase in GSH content and GST activity. Their results suggested that whey proteins may improve wound healing by the modulation of oxidative stress and antioxidant defense system. Furthermore, in the current study GST activity increased as a response to the increasing incubating time.

The obtained results showed that there was almost no effect of different pH values on whey immunoglobulins activity where their effect on GST enzymatic activity is still high at different pH. The explanation for this activity behavior may be due to the nature of immunoglobulins in whey proteins that resist acidic and basic conditions and still active at this different pH values. As camel has heavy chain, antibodies with antigen-binding regions consist of one single VH domain called VHH[13].

Conventional antibodies generally lose their binding ability after denaturation due to irreversible aggregation while the binding ability of many VHH domains is restored after denaturation due to their ability to refold[27]. Therefore, if these immunoglobulins were active at different pH values, they could affect the activity and stability of GST at these extreme pH conditions.

Regarding to the effect of pH on the GST enzymatic activity, enzymatic activity of GST was the highest at pH between 7 and 10. These results were in agreement with previous study that the purified GST from aerobic turtle liver had an optimum pH at 7.2[28]. The activity declined relatively slowly on the acidic side and fell sharply at higher pH values. In

addition to that, the optimum pH for silkworm GST with CDNB as a substrate was found to be 7.1[29]. In the presence of whey immunoglobulins, the activity of GST was increased sharply at pH values between 4 and 10. At pH 2, there was no effect of whey immunoglobulins on GST activity while at pH 12 whey immunoglobulins slightly increased GST activity.

Whey immunoglobulins were still active at different pH values and they significantly preserved GST pH stability. Despite that, at very high and very low pH values, GST enzyme may be denatured or subjected to conformational changes, so it was hard for immunoglobulins to protect GST at these harsh conditions.

Whey immunoglobulins were subjected to heat treatment to detect and observe impact on the effect of temperature on their activity on GST enzyme. The results showed the optimum activity of GST enzyme with whey immunoglobulins previously incubated at temperatures between (40-60) ºC. Moreover, the activity of these immunoglobulins at 70 ºC still present. This means that these immunoglobulins are stable at high temperatures up to 70 ºC. At very high temperatures the activity of whey decreased due to their instability which may relate to the denaturation of some proteins in whey at temperatures more than 63 ºC as found by Benabdelkamel and his collegues[30]. In their study, the activity of some proteins like lacto transferrin, Ig α-1 chain and serum albumin was decreased. Furthermore, Larson and Rolleri[31] found that heating skimmed milk at 70 ºC for 30 min resulted in a 89% loss in Ig activity.

Relating to the effect of temperature on purified sheep liver GST enzymatic activity, enzymatic activity of GST was the highest at temperatures between 20-30°C. These results were in harmony with the previous work which showed that the enzymatic activity of *Pseudomonas* GST was the highest at 30°C and decreased above 40°C[32]. Moreover, the silkworm GST enzymatic activity was increased with increasing temperature up to 25°C, then the activity started to decrease with increasing temperature[29].

In the presence of whey immunoglobulins, the stability of GST enzymes increased at higher temperatures than its activity alone up to 50°C. Meanwhile, the effect of whey immunoglobulins on enzyme activity reached zero at temperatures more than 60°C where there is no significant effect on the GSTs activity. The results can be explained by the fact that the high temperature caused denaturation for GST enzyme despite the presence of whey immunoglobulins. Whey immunoglobulins could not affect the thermal stability of GST after its denaturation or could not prevent enzyme denaturation.

In addition to that, the GSH kinetic was also studied. Camel whey immunoglobulins increased the Vmax for GSTs from 16.1 to 18.2 µmol/min/ml (13%) and increased Km from 0.025 to 0.031 mM (24%). When the Km value was increased, the affinity of GST to GSH was decreased and its capacity for GSH was increased. The current *in vitro* results were in accordance with the *in vivo* results obtained from a previous study on rate liver GST. The latest one showed that the farm and desert camel's milk increased Vmax and potentiate the catalytic efficiency of the GST enzyme[33].

Strategies to induce the activity of GSTs have been shown to protect against carcinogens in a variety of organs and across several species[34]. From the results obtained, the increased GST activity in the presence of camel whey immunoglobulins could protect cellular proteins against oxidation and detoxify reactive oxygen species generated from exposure to toxic xenobiotics.

Conclusion

The addition of whey immunoglobulins to the enzymatic reaction caused an overall increase in the activity of GSTs *in vitro*. So, further *in vivo* studies on the effect of camel whey immunoglobulins on the activity of GSTs are recommended. Moreover, those immunoglobulins can be modified and finalized in an industrial form to be used in health improvement.

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