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IN VITRO EFFECT OF CAMEL WHEY ON COPPER INACTIVATED GLUTATHIONE-S-TRANSFERASES

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Abstract

The main aim of the present study was to determine the in vitro effect of some heavy metals in general and copper in particular on the activity of sheep liver glutathione-s-transferases (GSTs). Besides that, the in vitro effect of camel whey on copper inactivated GSTs was also studied in this research. The effect of heavy metals zinc, nickel, cobalt, manganese, copper and camel whey on GSTs activity was studied by spectrophotometric method using 1-chloro-2,4-dinitro-benzene (CDNB) as substrate. Results showed that copper ion had the most potent inhibitory effect on GSTs activity when compared to other studied heavy metals as it directly reduced the enzyme residual activity to reach 29.3% at 2 mM concentration. In addition to that, the further increase in incubation time between heavy metals and GSTs lead to an increase in inhibitory behavior of all heavy metals under study. Furthermore, the role of camel whey on copper inactivated GSTs was clearly showed that it can reduced the inhibitory effect of copper on enzyme activity prior to its addition. The acquired residual activity after direct whey addition increased from 0.76% to 16.48% at 2 mM copper concentration. Moreover, the pre-incubation of GSTs with camel whey for a certain period caused better effect than its direct addition. Hence, the residual activity of GSTs increased from approximately zero to 38.5% at 2 mM. In conclusion, camel whey play an important role in the activation of copper inactivated GSTs.

INTRODUCTION

Glutathione-S-transferase isoenzymes are widely distributed in nature, being found in both eukaryotic and prokaryotic organisms [1]. The transferases possess different activities and participate in several different types of reactions. Most of these enzymes can catalyze the conjugation of reduced glutathione (GSH) with compounds that comprise an electrophilic centre through the formation of a thioether bond between the sulphur atom of GSH and the substrate [2]. In addition to the conjugation reactions, a number of GST isoenzymes display other GSH-dependent catalytic activities that involve the reduction of organic hydroperoxides and the isomerisation of different unsaturated compounds [3, 4]. These enzymes also have several non-catalytic functions including carcinogens isolation, intracellular transport of a wide spectrum of hydrophobic ligands, and modification of signal transduction pathways [5, 6]. Glutathione S-transferases represent two well-defined superfamilies that have transferase activity [7]. The first identified enzyme family is cytosolic which contains at least 16 members in humans [2, 7]. The soluble GSTs involved in the metabolism of foreign chemicals, such as carcinogens, environmental pollutants and cancer chemotherapeutic drugs, in addition to their role in the detoxification of endogenous harmful compounds [1]. Additionally, soluble glutathione-S-transferases have been considered to be one of phase II drug-metabolizing enzymes as its action follows phase I drug-metabolizing enzymes which is often cytochrome P450 (CYP) [8]. The CYP enzymes catalyse the introduction of a

functional group, such as an epoxide, into a chemically inactive xenobiotic. This functional group offers an electrophilic center that is attacked by reduced glutathione (GSH), the incoming nucleophile, in a reaction catalysed by GST. The addition of GSH to the molecule gives it a molecular 'flag' that allows the xenobiotic-conjugate to be removed from the cell. This process requires the participation of drug transporters such as multi-drug resistance associated protein (MRP) [9]. Moreover, some GSTs considered to serve antioxidant agents and others are involved in the synthesis and inactivation of prostaglandins [9, 10]. On the contrary, the second identified enzyme family is MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism). The members of this enzyme family are involved in the biosynthesis of leukotrienes, prostanoids and endogenous lipid signaling molecules rather than in the detoxification reactions [11].

Glutathione is one of the flushest thiol-containing molecules in animal cells and its function in the protection of tissues from the toxic effects of xenobiotics and endogenous electrophilic toxicants has been extensively studied [12]. Hepatic glutathione has a significant role in the biliary secretion of copper, zinc, methylmercuric, cadmium, and lead ions [13, 14, 15, 16]. Thus, the interaction of metals with glutathione is an integral part of the toxic response to many metals. Principally, copper ions are essential for electron transfer reactions in biological system. Even so, excess copper generates reactive oxygen species (ROS) via Fenton and/or Haber–Weiss reactions causing oxidative damage of biological molecules by inhibiting GSH regeneration [17]. Over and above, copper ions show high affinity for thiols and amino groups present in proteins. Their binding causes the alterations in the enzyme structure and hence inhibition of their activity [18]. Consequently, the oxidation by the generation of ROS and protein binding are two different mechanism causes GSH depletion as well as inhibition of several enzymes linked with GSH metabolism.

Camel milk was deemed as the main food for humanity because it has fundamental nutrients. On top of that, camel milk is better than other ruminant milk as it includes good qualities of lactoferrin, lysozyme, lacto-peroxidase and immunoglobulins [19]. Moreover, camel milk had many properties that make it very useful choice to be used in some parts of the world for healing certain diseases. In this aspect, whey proteins in camel milk had an important role as an antitumor and anti-carcinogenic agent [20]. In general, glutathione and glutathione-metabolizing enzymes have an important role in the preservation of mammalian cells against oxidative and alkylating agents [21]. Heavy metals are included in these agents and are used in many different industries. Accordingly, heavy metals are found in contaminated foodstuffs as well as water sources, so that the chance to offer these metals is high. Thus, the present work was designed to investigate the in vitro effect of camel whey on copper inactivated sheep hepatic GSTs.

MATERIALS AND METHODS

Glutathione-S-Transferase Preparation

The enzyme solution was prepared as the following: fresh sheep liver was washed, homogenized and centrifuged. Then the resulting supernatant that contains cytosolic glutathione-s-transferases was precipitated by ammonium sulfate at (30-70) %. After that, the obtained ammonium sulfate fraction was purified by gel filtration column chromatography using (Ultrogel ACA 44 column, Sigma). The protein levels for all fractions and GSTs activity for protein containing fractions were determined [22, 23]. Then, all fractions with GSTs were pooled and applied to affinity column (GSH-agarose, Sigma). After elution, all fractions were tested to determine GST activity and protein level [22, 23]. The fractions with GSTs were pooled, dialyzed, concentrated by freeze-drying to a concentration equal to 200 µg/ml and subsequently used for the activity studies.

Camel Whey Preparation

Camel whey sample was prepared as the following: the casein was precipitated from the skimmed milk sample based on milk renneting technique using commercially available rennin to obtain good crude contraction [24]. The coagulated milk was heated to 56°C for 10 min. Casein separation of lacto serum was carried out by filtration. For the final clarification, the lacto serum was again centrifuged (Sorval lynx 160) for 30 min at 10,000 rpm at 4°C. The pellet was discarded, and the supernatant was filtered using a Millipore filter (0.45µm), then the filtered supernatant was lyophilized to get powder of camel whey using (Millrock freeze dryer).

Glutathione-S-Transferase Activity Assay

Glutathione-S-transferase activity using one-chloro- 2, 4-dinitrobenzene (CDNB) as substrates was assayed spectrophotometrically using (Seacoman spectrophotometer) [23]. The cuvettes (final volume of 1.0 ml) contained 0.1 M phosphate buffer (pH 7), 1.5 mM GSH and 1.5 mM of CDNB and 50 µl of diluted enzyme (0.5 µg/ml). Change in absorbance at 340 nm was followed against a blank containing all reactants except CDNB activity was expressed as µmol conjugate formed/ min/ml using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Direct Effect of Heavy Metals on GSTs Activity

Different heavy metals ($ZnCl_2$, $NiCl_2$, $CoCl_2$, $MnCl_2$ and $CuCl_2$) were prepared at 2 mM concentration, then the effect of each heavy metal on the activity of GSTs enzyme was spectrophotometrically measured at 340 nm and expressed as residual activity. The residual enzyme activity is a ratio expressing relative enzyme activity (%) to the original untreated enzyme activity.

Effect of Heavy Metals Incubation Time on GSTs Activity

Heavy metals ($ZnCl_2$, $NiCl_2$, $CoCl_2$, $MnCl_2$ and $CuCl_2$) were prepared at 2 mM concentration and mixed with 0.5 µg/ml GSTs. Then all mixtures were incubated in ice at different time intervals (0, 30, 60 and 90 min). After that, the activity of GSTs was spectrophotometrically measured at 340nm.

Effect of Copper Different Concentration on GSTs Activity

Copper was prepared at various concentrations (0.5 ,1, 1.5, 2 mM) and mixed with 0.5 µg/ml GSTs. After that, the effect of each concentration on GSTs activity was measured at340nm spectrophotometrically.

Effect of Camel Whey on Copper Inactivated GSTs

Direct Effect of Camel Whey on Copper Inactivated GSTs

Glutathione-S-transferases were incubated for 30 min with (0.5, 1, 1.5 and 2 mM) copper ions. Then 250 µg/ml whey were directly added to the previous mixtures and its effect on copper inactivation was measured spectrophotometrically at 340 nm.

Incubation Time Effect of Camel Whey on Copper Inactivated GSTs

Half µg/ml GSTs were mixed with (0.5, 1, 1.5, 2 mM) copper ions and incubated for 30 min on ice. Then another 10 min incubation period after the addition of 250 µg/ml whey was done. The effect of whey on copper inactivated GSTs was assayed spectrophotometrically at 340nm.

Pre-Incubation Effect of Camel Whey on Copper Inactivated GSTs

Two hundred and fifty µg/ml whey were mixed with 0.5 µg/ml GSTs and (0.5, 1, 1.5, 2 mM) copper. All mixtures were incubated for 30 min on ice, after that the effect of incubated mixtures on the activity of GSTs was measured using a Seacoman spectrophotometer at 340nm.

RESULTS AND DISCUSSION

Heavy metals affect living organisms in different manners. As some heavy metals have essential functions and are toxic only in overdoses there is a need to maintain optimum levels of heavy metals inside the cells. To achieve that, living organisms use several metal detoxification mechanisms [25, 26]. Heavy metals affect the biological activity of a protein by their association with it. Therefore, the current in vitro metal toxicity experiment was performed in order to investigate the severity of heavy metal toxicity using purified sheep liver glutathione transferases (GSTs). The obtained results for the residual activity of glutathione-S-transferases after the addition of different heavy metals are shown in Figure 1. Results showed that copper ion had the most potent inhibitory effect on GSTs activity as it reduced the enzyme residual activity to reach 29.3%. On the other hand, the other studied heavy metals (Zn, Co, Ni, and Mn) demonstrated little inhibitory effect on the activity of GSTs when compared to the copper inactivation effect as they reduced the enzyme residual activity up to 89.3%, 97.3%, 96% and 86.7% respectively. Otherwise, the residual activity of glutathione-S-transferases enzyme after their incubation with several heavy metals for 30, 60 and 90 min are shown in Figure 2. Of great concern, results indicated that further increase in the incubation time between heavy metals and GSTs lead to an increase in the inhibitory behavior for all heavy metals under investigation. As the recorded residual activity after 90 min incubation for Zn (46.7%), Co (62.7%), Ni (52%) and Mn (56%). Furthermore, copper ions nearly abolished all GSTs activity (0.53% residual activity) within 90 min of incubation. Regarding to the protein level, a number of studies explicated how protein function and activity are affected by heavy metals. The inhibitory impact of heavy metals on proteins related to either metals are directly bound with proteins or metals generate reactive oxygen species (ROS) and elicit oxidative stress and so inhibit proteins biological activity [27, 28, 29].

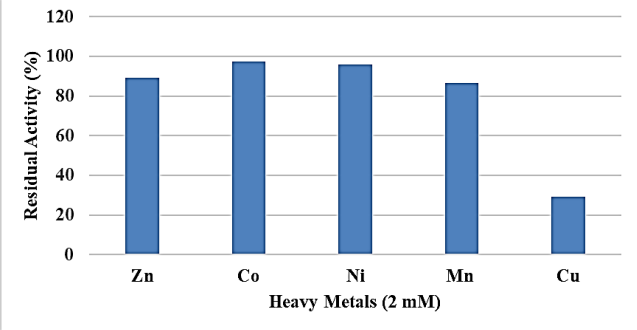

Figure 1. Direct effect of heavy metals on GSTs.

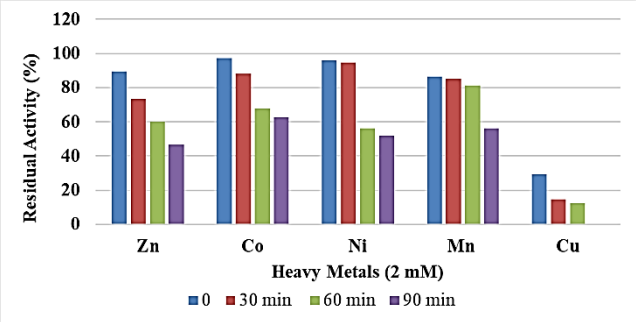

Figure 2. Effect of heavy metals incubation time on GSTs activity.

Based on the obtained results, the gradient increase in copper concentration lead to a decrease in GSTs activity as illustrated in Figure 3. It was clearly noticed that copper reduced GSTs activity significantly as 1, 1.5, 2 mM concentration of copper ions reduced the residual activity of GSTs up to 1.2% ,0.98% and 0.76%, respectively. The profound effect of copper was due to differences in its redox potentials compared to other metal ions. This difference leads to different toxicity mechanisms that underlie the damage promoted by these ions [30]. Copper ions generally act as co-factors of enzymes at low concentrations thus enhancing enzyme activity. But at sufficiently high concentrations, they can inhibit enzyme activity by two mechanisms. The first one is the involvement of reactive oxygen species (ROS) which are generated by Fenton and/or Harbor-Weiss reaction leading to oxidative damage to enzymes. The second one is a copper binding to thiol groups in protein and formation of incorrect intra and inter-subunit disulfide bonds between particular cysteine residues in proteins thus inhibiting its function [31]. The fundamental results of this research coincide with another study that evaluated the effect of monovalent, divalent, and trivalent cations on the camel liver GST activity and aggregation [32]. Their results showed that copper inhibited GST activity effectively compared to other tested metals. In the former mentioned research, GST inhibition was due to the high affinity binding of copper with enzyme –SH groups [33]. This binding altered the conformation of the enzyme due to which the enzyme loses activity, and intermolecular affinity increases, which leads to the formation of higher-order aggregates by the oxidation of intermolecular thiol groups. From the obtained results, sheep liver GSTs are not exception to the GSTs of the other organisms in terms of their behavior under the influence of copper.

The evaluation of the in vitro effect of camel whey on copper inactivated GSTs was represented in Figure 4, 5 and 6. Direct addition of camel whey provides that the inhibitory effect of copper ions was reduced at all studied copper concentrations when compared to controls without camel whey (Figure 4). The acquired residual activity after direct whey addition increased from 0.76% to 16.48% at 2 mM, from 0.99% to 17.14% at 1.5 mM, from 1.21% to 21.43% at 1 mM and from 19.1% to 38.46% at 0.5 mM copper concentration. Moreover, the effect of incubation time between copper inactivated GSTs and camel whey was also studied (Figure 5). It was concluded that further incubation periods displayed better activation for copper inactivated GSTs when compared to the direct activation effect of camel whey. Regarding that, the residual GSTs activity increased to 37.4%, 47.25%, 58.4% and 63% at 2, 1.5, 1 and 0.5 mM respectively. In addition to that, the pre-incubation of GSTs, copper ions and camel whey showed the best activation effect of the enzyme as the residual activity of GSTs increased from approximately zero to 38.5% at 2 mM concentration in the presence of camel whey (Figure 6). As previously mentioned, results showed a decrease in GSTs

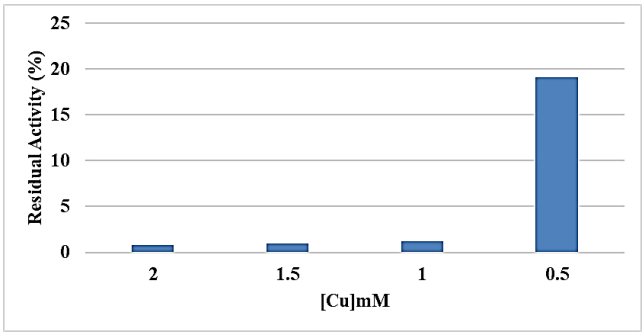

Figure 3. Effect of copper different concentration on GSTs activity.

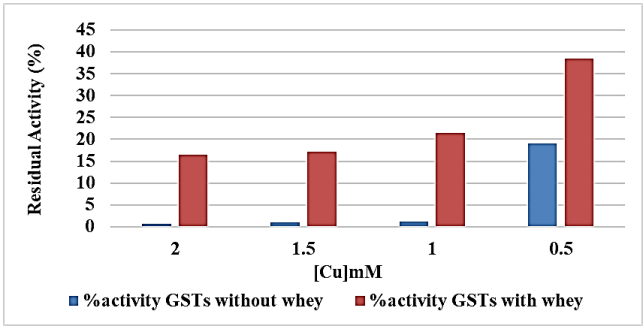

Figure 4. Direct effect of camel whey on copper inactivated GSTs.

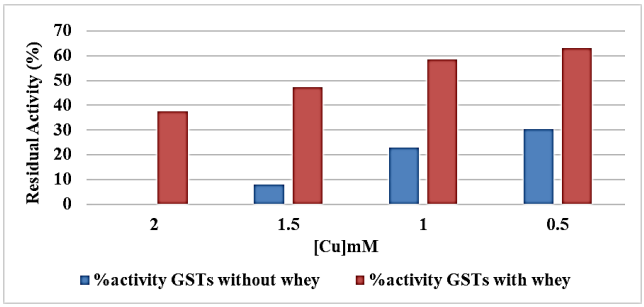

Figure 5. Incubation time effect of camel whey on copper inactivated GSTs.

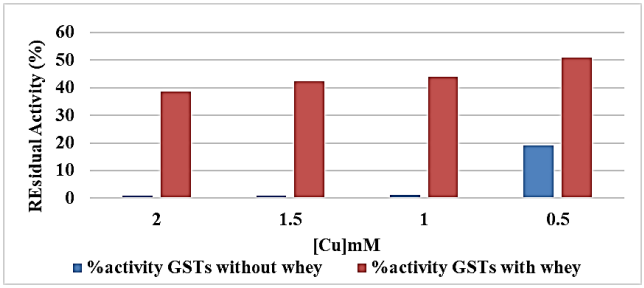

Figure 6. Pre-incubation effect of camel whey on copper inactivated GSTs

inactivation by copper in the presence of camel whey. This reactivation effect is mostly due to the direct activation of GSTs by immunoglobulins found in camel whey as reported by the previous study, which demonstrated the effect of camel whey immunoglobulins on GSTs activity [34]. In their experiment, whey immunoglobulins concentrate affected GSTs activity positively in direct relationship even in harsh conditions. This may explain the reduction of copper inactivation influence on GSTs in the presence of camel whey. Moreover, analysis of camel whey constituents showed the presence of two other proteins which are lactoferrin and albumin [35]. Both proteins possess a binding site for copper [36, 37]. Therefore, their binding ability to copper may lead to the alleviation of copper inhibitory effect on GSTs in the current study.

In conclusion, this in vitro effect of camel whey on copper inactivated GSTs brings up several questions; can this also be applied in vivo? Can camel whey be used as prophylactic or therapeutic substrate, especially for areas depending on high copper concentration water sources? and is this effect specific to camel whey only? Further studies should be done to answer these questions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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