



Correlation between the level of sulfane sulfur and the expression/activity of sulfurtransferases in chicken tissues – a possible ways of cyanide detoxification

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Abstract

Cyanogenic glycosides are a large group of secondary metabolites that are widely distributed in the many plants commonly consumed by humans, birds, and other animals. Thiosulfate sulfurtransferase (TST) and 3-mercaptopyruvate sulfurtransferase (MPST), are two evolutionary-related enzymes that constitute the defense against cyanide toxication and participate in the production of sulfane sulfur-containing compounds. The expression and activity of TST and MPST as well as the level of sulfane sulfur in chicken tissue homogenates of the liver, heart, and gizzard were investigated. The highest expression/activity of TST and MPST was noticed in liver homogenates which was associated with the high sulfane sulfur level. Both the expression and activity of TST as well as the sulfane sulfur level in chicken gizzard homogenates were significantly lower than in the liver and heart. Both TST and MPST enzymes can play an important role in cyanide detoxification in chicken tissues. Maintaining appropriate sulfane sulfur level together with the high activity of these enzymes is essential to protect tissues from the toxic effects of cyanide, released from certain nutrients.

Keywords Cyanide detoxification · Cyanogenic glycosides · Thiosulfate sulfurtransferase · 3-Mercaptopyruvate sulfurtransferase · Sulfane sulfur

Introduction

Cyanide is a highly toxic compound that binds to ferric ion (Fe^{3+}) of cytochrome oxidase (complex IV), a key enzyme of the respiratory chain and inhibits oxidative metabolism, which may eventually lead to cell death (Egekeze and Oehme 1980). The cyanogenic glycosides (cyanide-containing

precursors) are widely distributed in the environment. Studies have shown the presence of cyanogenic glycosides in more than 2000 plant species, which are commonly included in the diets of humans, avians, and other animals (Bolarinwa et al. 2014; Nyirenda 2020). Table 1 shows the most common ones. The potential toxicity of cyanogenic glycosides and their derivatives largely depends on their ability to release hydrogen cyanide (Nyirenda 2020).

Many studies report the death or poisoning of animals that have ingested cyanogenic plants (Wiemeyer et al. 1986). At the turn of the last few years, it has become a popular practice to add cassava flour to feed as a substitute for maize, thereby significantly reducing production costs. The literature confirms the safe use of cassava as a feed additive for poultry, although estimating the maximum level in rations is a problem. Cyanide levels in cassava range from 75 to 1000 mg/kg, depending on variety, age, and environmental factors (Ngiki 2014). Cyanide hydrolysis is mediated by two enzymes- β -glucosidase and hydroxynitrile lyase present in the gastrointestinal tract of animals (Fomunyan et al. 1984; Gonzales and Sabatini 1989).

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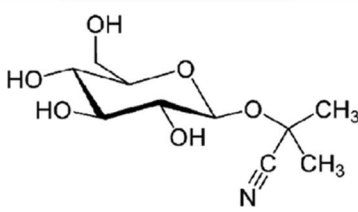
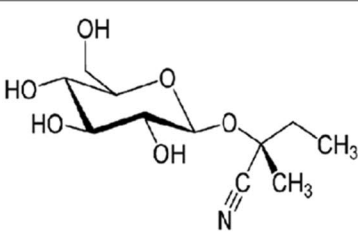
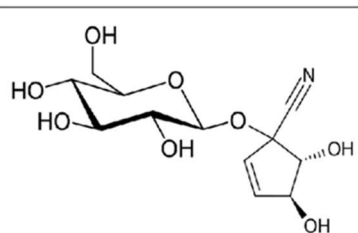
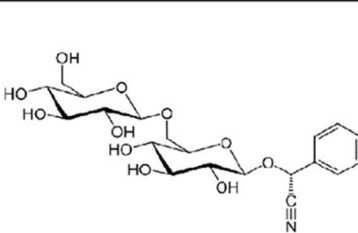
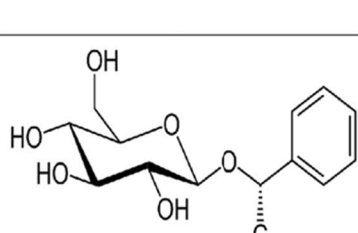
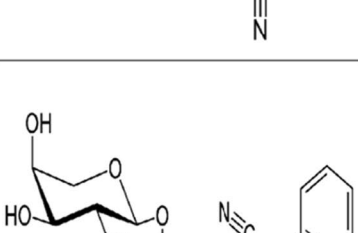
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Table 1 The most common cyanogenic glycosides among plant species (Francisco and Pinotti 2000; Vetter 2000; Bjarnholt and Møller 2008)

SUBSTITUENT TYPE	CYANOGENIC GLYCOSIDES	CHEMICAL FORMULA	SPECIES	VEGETATIVE PART	
Aliphatic substituent	Linamarin		<i>Manihot esculenta</i> <i>Trifolium repens</i> <i>Linum usitatissimum</i>	Leaf	
		Lotaustralin		<i>Manihot esculenta</i> <i>Phaseolus lunatus</i> <i>Linum usitatissimum</i>	Bark Seed Leaf
			Gynocardin		<i>Ceratosicyos laevis</i> <i>Rawsonia lucida</i> <i>Ryparosa kurrangii</i>
Aromatic substituent	Amygdalin			<i>Prunus amygdalus</i> <i>Prunus avium</i> <i>Prunus armeniaca</i>	Seed
		Prunasin		<i>Prunus laurocerasus</i> <i>Prunus perlica</i> <i>Prunus armeniaca</i> <i>Prunus avium</i>	Leaf Seed
			Vicianin		<i>Vicia sativa</i> <i>Gerbera jamesonii</i> <i>Davallia trichomanoides</i>

Cyanide detoxification mechanisms are based on reactions catalyzed by sulfurtransferases (Aminlari and Shahbazi 1994). There are two main, evolutionary close-related enzymes responsible for that action: thiosulfate sulfurtransferase (TST, rhodanese, EC 2.8.1.1) and 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2). In numerous studies, their pivotal role was emphasized due to constituting the fundamentals of anti-cyanide defense in living organisms (Aminlari and Shahbazi 1994; Nagahara et al. 1999; Wróbel et al. 2004; Agboola et al. 2006; Kruithof et al. 2020; Pedre and Dick 2021; Zuhra and Szabo 2022). The reactions catalyzed by these enzymes are illustrated in Fig. 1.

Thiosulfate sulfurtransferase is a mitochondrial enzyme, whereas 3-mercaptopyruvate sulfurtransferase is present in the cytosolic and mitochondrial cell compartment (Pallini et al. 1991). The presence of TST was confirmed in animals, plants, fungi, bacteria, and archaea (Kohanski and Henrikson 1990; Aminlari and Shahbazi 1994; Agboola et al. 2006; Aminlari et al. 2007; Cipollone et al. 2007; Wang et al. 2020). The distribution of the MPST appears to be more complex, as the MPST gene seems not to be universally conserved. The lack of MPST concerns archaea, although its presence is patchy within eubacteria, which means that it could be found in some branches of the eubacterial phylogenetic tree. In the eukaryote domain, MPST exists in most phyla of animals, plants, and fungi. MPST is present in all vertebrates, but this enzyme in lower organisms (e.g. tunicates and most insects) is of minor importance and does not appear to be essential (Agboola et al. 2006; Pedre and Dick 2021). The sulfurtransferases also play other significant roles in maintaining cellular homeostasis. There is evidence that rhodanese is involved in the formation of iron-sulfur centers, participation in energy metabolism, and functioning as thioredoxin oxidase (Aminlari et al. 1997, 2007; Agboola et al. 2006).

The proper activity of these sulfurtransferases is especially important in animals at higher risk of cyanide poisoning, mainly by consuming plants abundant in cyanogenic glycosides (Ejiro and Ogheneovo 2015).

Sulfurtransferases such as TST and MPST, but also other enzymes like cystathionine γ -lyase (CTH) and

cystathionine- β -synthase (CBS) participate in the formation of sulfane sulfur-containing compounds (Toohey and Cooper 2014). Sulfane sulfur is a common cellular component and is present in several forms, including persulfides, polysulfides, thiosulfate, and elemental sulfur (Toohey 2011; Shinkai and Kumagai 2019). MPST, CTH, and CBS can also produce hydrogen sulfide (H₂S), which is oxidized by TST to thio-sulfate (Stipanuk and Ueki 2011; Kimura 2021).

The aim of our study was to assess the correlation between the level of sulfane sulfur and both the expression and activity of sulfurtransferases in various tissue homogenates of the chicken which presumably have a higher risk of exposure to cyanide or its precursors, because of the type of ingested food. In this paper, both the expression and activity of TST and MPST as well as the sulfane sulfur levels in chicken liver, heart, and gizzard homogenates were investigated. We believe that our research will allow a better understanding of the importance of sulfurtransferases in maintaining the sulfane sulfur pool at an appropriate level, which may be important in toxicology.

Materials and methods

Tissue homogenization

The chicken tissues (liver, heart, gizzard) obtained from a local market in Kraków (Poland) were weighed and homogenized in ice-cold 0.1 M phosphate buffer pH 7.5 (1:4, w/v) using a blender homogenizer. After centrifugation at 1600 × g at 4 °C for 10 min, the supernatant was used immediately for determination of sulfurtransferases activity, sulfane sulfur level, and protein content.

Sulfane sulfur level

The level of sulfane sulfur was determined by the method of Wood (1987), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion. The data were expressed as μ moles of SCN[−] produced per 1 mg of protein.

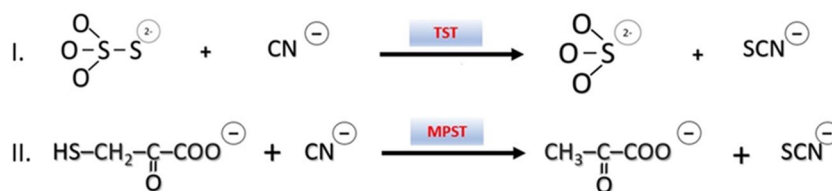


Fig. 1 Cyanide detoxification catalyzed by thiosulfate sulfurtransferase and 3-mercaptopyruvate sulfurtransferase. The first reaction catalyzed by TST between thiosulfate and cyanide is resulting in thiocyanide formation. The second reaction catalyzed by MPST shows

the formation of thiocyanide with 3-mercaptopyruvate as one of the substrates. Created based on References (Egekeze and Oehme 1980; Westley 1981; Aminlari and Shahbazi 1994; Aminlari et al. 1997; Al-Qarawi et al. 2001; Ejiro and Ogheneovo 2015)

Protein content

The total protein content was determined by the method of Lowry et al. (1951). The solution of bovine serum albumin was used as a standard. The stock solution of bovine serum albumin (1 mg/ml) was used to prepare serial dilutions to create a standard curve.

Total RNA isolation

Total RNA was extracted from the chicken tissues (liver, heart, gizzard) using TRIzol reagent according to the manufacturer's instructions (Invitrogen, CA, USA). Quantification and purity of RNA extracted from each sample was done using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was further assessed by resolving the 28S and 18S ribosomal RNA bands on the agarose gel.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of *Tst*, *Mpst*, and *β-actin* genes was analyzed in chicken tissues. Reverse transcription was performed using the NG-dART RT kit (E0801-01, EURx, Gdańsk, Poland) according to the manufacturer's instructions. Amplification of cDNA samples was performed using the Color OptiTaq PCR Master Mix (2x) kit (E2915-01 EURx, Gdańsk, Poland) according to the manufacturer's instructions.

The PCR conditions for the *Tst*, *Mpst* and *β-actin* genes in chicken tissues are published for the first time in this paper.

The PCR cycling conditions for the *Tst*, *Mpst*, and *β-actin* genes were configured as follows: 94 °C (5 min) for initial denaturation; denaturation 94 °C (30 s), annealing phases at specific temperatures for each gene — 59 °C for *Tst* (30 s), 61.2 °C for *Mpst* (30 s), and 55.8 °C for *β-actin* (30 s), elongation 72 °C (1 min) for 28 cycles with a final extension at 72 °C (8 min).

The corresponding primer sequences were as follows:

For *Tst*: forward 5'-AGGCGGTCTTTAAGCCAAG-3', and reverse 5'-TTCTCATGGCCGCTTTCTGT-3' (214 bp),

For *Mpst*: forward 5'-ACGAGTGTGGTGGATGTTCC-3', and reverse 5'-GTGAAGGGCATGTTTCAGGGA-3' (309 bp),

For *β-actin*: forward 5'-TGCTGACAGGATGCAGAA GG-3', and reverse 5'-ACAGGGAGGCCAGGATAG AG-3' (122 bp).

The mRNA sequences for *Tst*, *Mpst*, and *β-actin* genes were procured from the National Center for Biotechnology

Information. *β-actin* was utilized as an internal standard to normalize all the samples. *β-actin* gene showed a constant expression levels between examined chicken tissues for each independent experiments.

After amplification, the PCR products were resolved in a 2% agarose gel containing ethidium bromide. The gels with amplification fragments were visualized and photographed under UV light using the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Sulfurtransferases activity

TST activity was assayed by the Sörbo (1995) method, following a procedure described by Wróbel et al. (2004). TST activity was expressed as $\mu\text{moles SCN}^-$ formed during 1 min incubation at 20 °C per 1 mg of protein. MPST activity was assayed according to the method of Valentine and Frankenfeld (1974), with some modifications as described by Wróbel et al. (2004). MPST activity was expressed as μmol of pyruvate produced during 1 min incubation at 37 °C per 1 mg of protein.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA). The results were presented as the means \pm standard deviations (SD). Data were analyzed by Student's t-test or the Mann–Whitney U test. The differences were considered statistically significant at $p < 0.05$. All the experiments were repeated at least three times.

Results

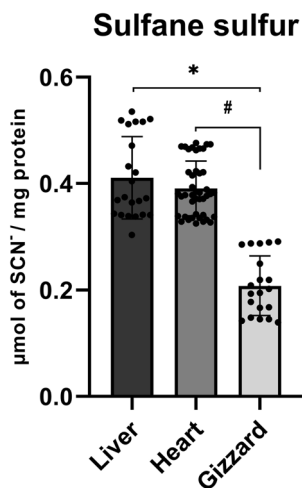
Sulfane sulfur level in chicken tissue homogenates

Our studies demonstrated the high level of sulfane sulfur in the liver and heart. In turn, the sulfane sulfur level was significantly lower in the chicken gizzard (Fig. 2).

Expression of thiosulfate sulfurtransferase and 3-mercaptopyruvate sulfurtransferase genes in chicken tissue homogenates

The expression of *Tst* and *Mpst* genes in different chicken tissue homogenates was studied on the mRNA level (Fig. 3a, b). We observed that the expression of *Tst* and *Mpst* genes was significantly highest in the liver as compared to the heart and gizzard. The representative results of these studies are shown in Fig. 3a.

Fig. 2 The level of sulfane sulfur in chicken tissue homogenates. The results are expressed as the average \pm SD of three or more independent experiments. Significant statistical differences are presented respectively as * ($p < 0.0001$) between the level of sulfane sulfur in the liver and gizzard; # ($p < 0.0001$) between the results in heart and gizzard



The activity of thiosulfate sulfurtransferase and 3-mercaptopyruvate sulfurtransferase in chicken tissue homogenates

The results show statistically significant differences in the activity of TST and MPST in chicken tissue homogenates. Both enzymes exhibited their highest activity in the liver followed by the heart and respectively gizzard (Fig. 4).

Discussion

In this paper, for the first time, the level of sulfane sulfur in chicken liver, heart, and gizzard homogenates was investigated. We have shown that the level of sulfane sulfur was the lowest in the investigated chicken gizzard homogenates, as compared with the chicken liver and heart homogenates, which was related to the low expression and activity of MPST as well as TST in the chicken gizzard homogenates.

Previous articles indicated that the activity of rhodanese is higher in the chicken liver compared to other tissues (Aminlari et al. 1997, 2007; Al-Qarawi et al. 2001). In this paper, we have found that both the expression of *Tst* and the activity of TST were significantly higher in the liver homogenates compared to examined chicken tissues (such as the heart and the gizzard). We also observed high expression of *Tst* as well as the activity of TST in heart homogenates. High TST activity in the heart (Aminlari and Shahbazi 1994; Agboola et al. 2006; Eskandarzade et al. 2012) might reflect

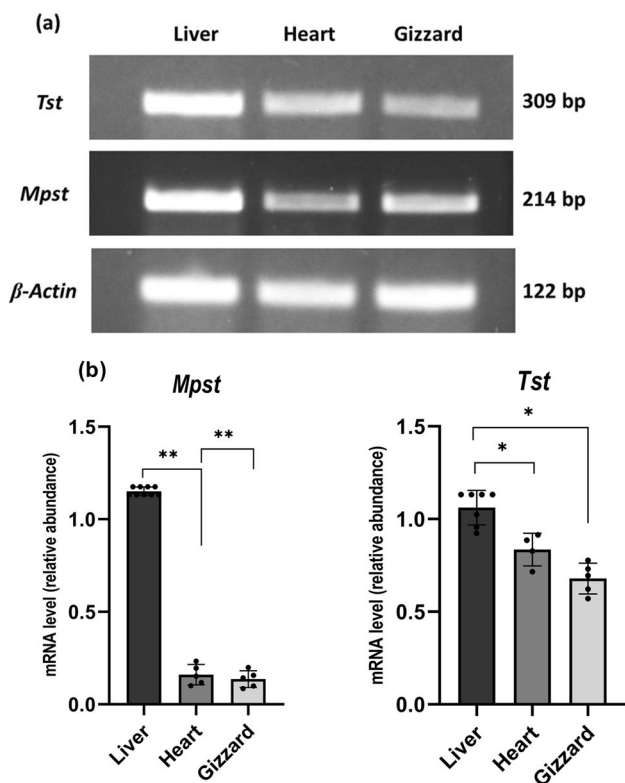


Fig. 3 a The expression of *Tst*, and *Mpst* on the mRNA level in chicken tissue homogenates. β -actin was used as the loading control. Data from a representative experiment are presented. All experiments were repeated at least three times with analogous results. b Densitometry analysis of *Tst* and *Mpst* genes expression in chicken tissue homogenates. Quantitative *Tst* and *Mpst* gene expression data are normalized to the expression levels of the β -actin gene (as the loading control). The results are expressed as the mean \pm SD of three or more independent experiments. Significant statistical differences are presented respectively as * (indicating $p < 0.05$) and ** (indicating $p < 0.0005$) between mRNA expression levels in the liver and the following tissue

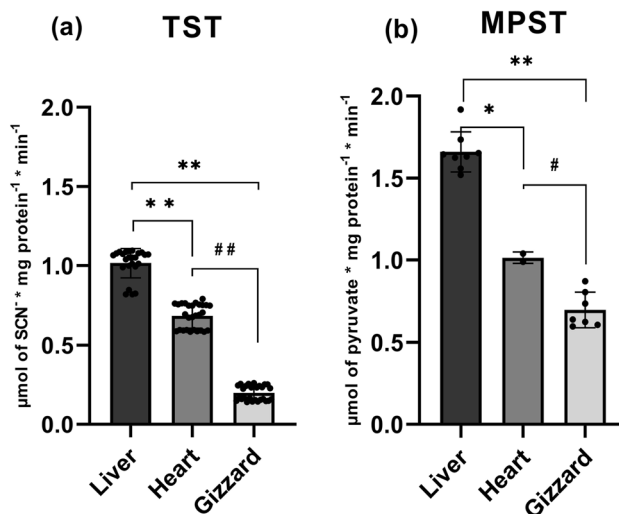


Fig. 4 The activity of TST (a) and MPST (b) enzymes in chicken tissue homogenates. The results are expressed as the average \pm SD of three or more independent experiments. Significant statistical differences are presented respectively as * (indicating $p < 0.05$) and ** (indicating $p < 0.0001$) between activity in the liver and the following tissue; # ($p < 0.05$) and ## ($p < 0.005$) between the results in heart and gizzard

the energy demands of this tissue that are provided mostly by aerobic pathways. It is known that rhodanese serves as a sulfur donor to enzymes and iron-sulfur clusters of the mitochondrial respiratory chain (Ogata and Volini 1986; Ryzd et al. 2021). The level of sulfane sulfur and H_2S is substantially reduced in cardiovascular diseases (Rajpal et al. 2018; Kolluru et al. 2022). Interestingly, the administration of sodium thiosulfate (a substrate for TST, and a donor of hydrogen sulfide) to protect the heart from "ischemia–reperfusion injury" is currently underway (2018–2023) in patients with myocardial infarction and/or heart failure (Kolluru et al. 2022).

The activity of TST in the gizzard of chicken is low (Oh et al. 1977; Aminlari et al. 1997; Agboola et al. 2006; Eskandarzade et al. 2012). In our paper, we have shown that both the expression of *Tst* and the activity of TST in chicken gizzard homogenates were significantly lower than in the homogenates of the liver and heart.

Interestingly, Aminlari et al. (1994; 2007) observed that in the chicken tissues, there is a higher activity of rhodanese in the epithelium of the different parts of the digestive system than in the liver. It is considered an adaptation to higher direct exposure to cyanogenic glycosides that liberate cyanide particles after the enzymatic action of the digestive tract microbiome (Ejiro and Ogheneovo 2015). Egekeze and Oehme (1980) demonstrated that thiocyanate concentration was significantly higher in the serum, sections of the digestive tract, and some organs of chickens given cyanide directly compared with those given cyanide-contaminated feed. In the digestive tract, the highest concentrations of thiocyanate (a product of TST and MPST activity) have appeared in the cloaca, where the metabolic waste is mixing with urine, and in the proventriculus, because here the digestion de facto begins and it probably provides cyanide detoxification before it reaches the general circulation. From other tissues, the highest SCN^- concentration was noticed in the liver, heart, and pancreas, as well as in the serum (Ejiro and Ogheneovo 2015; Ng et al. 2021) indicated that oral administration of sodium thiosulfate (a sulfur-donor substrate for TST) improved survival, blood pressure, respiration, and blood lactate concentrations in a large swine model of acute oral cyanide toxicity.

In this study, we observed that both expression and activity of MPST in the chicken liver homogenates were significantly higher compared to heart and gizzard homogenates. Moreover, we noticed that the activity of MPST in the liver homogenates was higher than the activity of TST. Previous results of Frendo and Dudek (1978) showed that MPST activity in the liver of developing chick embryos was also higher than TST activity. Mousa and Davis (1991) reported that urinary excretion of thiocyanate was increased in chicken after administration of 3-mercaptopyruvate (a substrate for MPST), but thiosulfate (a substrate of TST) did

not affect the amount of the released thiocyanate. Therefore, it seems that MPST is the main enzyme involved in the detoxification of cyanide in chicken tissues. Agboola et al. (2006) report that in poultry chickens the MPST activity (in the cytosolic fraction) was highest in the kidney, liver, and lung, and was low in the heart. Recent studies (Jin et al. 2020) have shown that the endogenous production of H_2S in chicken erythrocytes (which contain mitochondria) was mainly catalyzed by MPST. Wu et al. (2016) reported that chickens fed with a low-selenium diet exhibited lower expression of H_2S -producing enzymes (3-mercaptopyruvate sulfurtransferase, gamma-cystathionase, and cystathionine beta-synthase), and lower H_2S production in small intestinal tissues, whereas the expression of intestinal inflammatory factors was up-regulated. MPST activity is regulated by NADPH-dependent thioredoxin reductase/thioredoxin system; thioredoxin reductase is one of the enzymes which contains selenocysteine in its active site (Nagahara 2018).

Previous studies have shown that the deficiency of vitamin B6 (a precursor of pyridoxal phosphate- PLP coenzyme) and sulfur amino acids were associated with the severity and incidence of gizzard erosion in chickens (Miller et al. 1975; Dagher and Haddad 1981; Gjevre et al. 2013). It is known that cysteine aminotransferase (which produced 3-mercaptopyruvate as a substrate for MPST), CBS, and CTH are PLP-dependent enzymes involved in L-cysteine metabolism (Hipólito et al. 2020; Zuhra et al. 2020). The sulfane sulfur can be highly nucleophilic and plays an essential role in protection against electrophile toxicity- a covalent modification of cellular proteins associated with its dysfunction. The binding of sulfane sulfur to electrophiles forms sulfur adducts as detoxified metabolites (Shinkai and Kumagai 2019). The knockdown or knockout of sulfane sulfur-producing enzymes results in increased electrophile toxicity induced by methylmercury (Yoshida et al. 2011), cadmium (Akiyama et al. 2017), or acetaminophen (Hagiya et al. 2015). Thus, exogenous sulfane sulfur donors could be used as a chemopreventive agent against electrophilic stress (Shinkai and Kumagai 2019).

Conclusions

In the investigated chicken tissues the level of sulfane sulfur is relatively high. MPST is crucial for maintaining the appropriate level of sulfane sulfur and for cyanide detoxification, which can be judged based on higher values of specific activities, compared to TST, in all examined tissues. It is known that the activity of these enzymes can be dependent on the administration of e.g. sodium thiosulfate (as a substrate for TST), vitamin B6 (for PLP-dependent cysteine aminotransferase-CAT as a component of the CAT: MPST axis), N-acetylcysteine (a precursor of L-cysteine) or

3-mercaptopyruvate (a direct substrate for MPST); therefore, supplementation with these compounds can be an important factor in preventing the toxic effects of cyanide.

Author contributions Conceived and designed the experiments: H.J. Performed the experiments: A.M.; K.K.; H.J. Analyzed the data: L.R.; A.M.; H.J. Wrote the paper: H.J.; K.K.; A.M. Review, discussion, and editing: H.J. and M.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Available upon request.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest The authors declare no conflict of interest.

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