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OPTIMIZATION OF ENZYMATIC ASSAY OF SUPEROXID DISMUTASE AND GLUTATION PEROXIDASE ACTIVITY IN MILK WHEY

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Abstract
General spectrofotometric kinetic protocols are described to measure the antioxidant enzyme activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in milk whey. The SOD convert superoxide radical into hydrogen peroxide and molecular oxygen, while the GPx convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide, are converted to the harmless product water. The applicability of this methods for milk samples from dairy cows were tested. SOD activities in the milk whey was determined by the autoxidation of pyrogallol in presence of DTPA in TRIS-HCl buffer, pH= 8.5. The effect of reaction conditions on enzymatic inhibition of pyrogallol autooxidation was studied when different amounts of sample were added in reaction mixture. The reaction mixture for determination of GPx activity contained EDTA, reduced glutathione, glutathione reductase, NADPH and cumene hydroperoxide in phosphate buffer, pH= 7.6. The effect of reaction conditions on enzymatic NADPH consumption was studied when different amounts of reduced glutathione and whey sample were added in reaction mixture. The both spectrophotometric methods were modified in order to accommodate kinetic analyses in 96-well micro plates. The rate of pyrogallol autooxidation and NADPH oxidation linearly depend from amount of whey sample in reactive mixture. Applying higher amounts of milk whey in reactive mixture was decreasing optical density reading of pyrogallol autooxidation at 415 nm and increasing the optical density readings of NADPH oxidation at 340 nm, in a linear way. The methods could be validly applied for milk whey samples.
Key words: dairy cows, milk whey, superoxid dismutase, glutathion peroxidase.

Introductıon

Reactive oxygen species (ROS) are produced in many aerobic cellular metabolic processes. They include, but are not limited to, species such as superoxide and hydrogen peroxide which react with various intracellular targets, including lipids, proteins, and DNA (Bandyopadhyay et al., 1999). Although ROS are generated during normal aerobic metabolism, the biological effects of ROS on these intracellular targets are dependent on their concentration and increased levels of these species are present during oxidative stress.

Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways (Halliwell and Gutteridage, 2007). Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for life in all oxygen metabolizing cells are superoxide dismutase (SOD), catalase, and a substrate specific peroxidase, glutathione peroxidase (GPx) (Halliwell, 1987). The SOD catalyze the dismutation of the superoxide radical anion into oxygen and hydrogen peroxide, while the catalase and peroxidases convert hydrogen peroxide into water and in the case of catalase to oxygen and water (Bordo et al., 2000). The net result is that two potentially harmful species, superoxide and hydrogen peroxide, are converted to water.

The oxidative stability of milk is a balance between pro- and anti-oxidative factors (Lindmark-Mansson and Akesson, 2000); hence, identification and characterization of these factors is necessary to predict the rate of oxidation. A number of low-molecular weight antioxidants have been described in milk, e.g., ascorbate, urate and atocopherol, to mention the most important as judged from their abundance and consumption during oxidation (Nielsen et al., 2001). However, milk also contains antioxidative enzymes, such as catalase, superoxide dismutase and glutathione peroxidase (GPx) that can reduce oxidative deterioration, but the importance and relative contributions of these enzymes to the oxidative stability of milk is unclear (Lindmark-Mansson and Akesson, 2000).

The indirect spectrophotometric assay has been developed for measuring SOD and GPx activity in plasma, erythrocyte lysates, tissue homogenates and cell lysates (Paglia and Valentine, 1967; Marklund and Marklund,1974).

To some extent, the limited knowledge of the role of SOD and GPx in milk may be explained by the lack of suitable activity assays. Spectrofotometric kinetic assays were already successfully been applied for blood samples, we aimed to establish this tests for milk samples to evaluate it's relationship with occurrence of oxidative stress in dairy cows and herein report the limitations we observed for the application of the assays in milk.

Materıals and methods

Milk (raw, i.e. unhomogenized and unpasteurized) was obtained from private dairy farm. Milk was defatted by centrifugation at 5000 rpm for 20 minutes at 4^0C, and skim milk beneath the cream was gently poured into a clean container. Acid whey was prepared by treating the samples with 1M HCl to pH 4.6, followed by centrifugation (5000 rpm for 20 min at 4^0C) in order to remove precipitated proteins. The pH was adjusted usually to pH 7.6 using 1M NaOH. The samples were stored in stoppered plastic tubes at -80^0C. Before use the aliquots were thawed.

SOD activities in the milk whey was determined by the autoxidation of pyrogallol in presence of DTPA in TRIS-HCl buffer, pH= 8.5. The assay medium in a total volume of 200 µl contained 100 mM Tris HCl buffer, pH 8.5, 1mM DTPA (diethylenetriaminepentaacetic acid) in presence of 0.2 mM of pyrogallol. Pyrogallol (1,2,3-benzenetriolo) was dissolved in 10 mM HCl to obtain stock solution (2 mM) which was prepare always fresh. Measurement of SOD activity was carried out spectrophotometrically at 25^0C by the method of Marklund and Marklund (1974) with some modifications in order to accommodate kinetic analyses in 96-well microplates (Gao et al.,1998). The reading of absorbance at 415 nm for 3 minutes was made in interval of 60 seconds after an induction period of some 10 seconds. The bovine Cu/Zn-SOD was used as

standard for generation of a standard curve, a range activity of 100 U/ml to 0.09765 U/ml. The values obtained for SOD activity in the milk sample were then calculated from the standard curves using logarithmic regression and were finally expressed as percentage of inhibition of the pyrogallol autoxidation rate. One unit of the enzyme activity is defined as the amount which produced 50% inhibition of pyrogallol autoxidation under the standard assay conditions. The effect of reaction conditions on enzymatic inhibition of pyrogallol autooxidation was studied when different amounts of sample were added in reaction mixture.

The principle of the coupled enzymatic assay first described by Paglia and Valentine (1967) and modified according Chen et al. (2000) in order to accommodate kinetic analyses in 96-well microplates. The reaction mixture for determination of GPx activity in a total volume of 200 µl contained 50 mM potassium phosphate buffer, pH= 7.6., 5 mM EDTA (ethylenediaminetetraacetic acid), reduced glutathione (GSH), 1 U/ml glutathione reductase (GR), 0.25 mM NADPH. The measurements were carried out at two different concentrations of GSH i.e. 2 mM and 1 mM. Then cumene hydroperoxide (cH_2O_2) was added to initiate the reaction in a total volume of 30 µl at 37^0C. Stock solution of cumene hydroperoxide (9.9 mM) in 50 mM phosphate buffer, pH= 7.6. was prepare always fresh. The effect of reaction conditions on enzymatic NADPH consumption was studied when different amounts of reduced glutathione (GSH) and whey sample were added in reaction mixture. The assay temperature was 37^0C. The reading of absorbance was made at 340 nm for 3 minutes in interval of 60 seconds after an induction period of some 10 seconds. One unit of GPx was defined as the activity that causes the formation of 1,0 µmol/L $NADP^+$ from NADPH per minute at pH 7.6 at 37^0C in a coupled reaction.

All chemicals were of the highest purity grade. TRIS, pyrogallol, KH_2PO_4, EDTA and cH_2O_2 were purchased from Merck, Darmstadt, Germany. DTPA, Cu/Zn-SOD, GSH, NADPH and GR were obtained from Sigma Chemical Co., St. Louis, MO, USA. The absorption was determined with a spectrophotometer Bio-Rad 680 XR, microplate reader.

Results

The standard curves demonstrated a linear relationship between absorption measurements and the SOD activity. The colored dye is directly proportional to the percentage of the pyrogallol autoxidation rate. Since the rate change of absorbance per minute at 415 nm is proportional with the amount of superoxide anion, the SOD activity in reactive mixture as an inhibition activity of pyrogallol autooxidation is proportional with decrease in color development at 415 nm. Therefore, the increasing of absorbance at 415 nm (A_{415}) per minute followed a linear kinetics, showed on Figure 1.

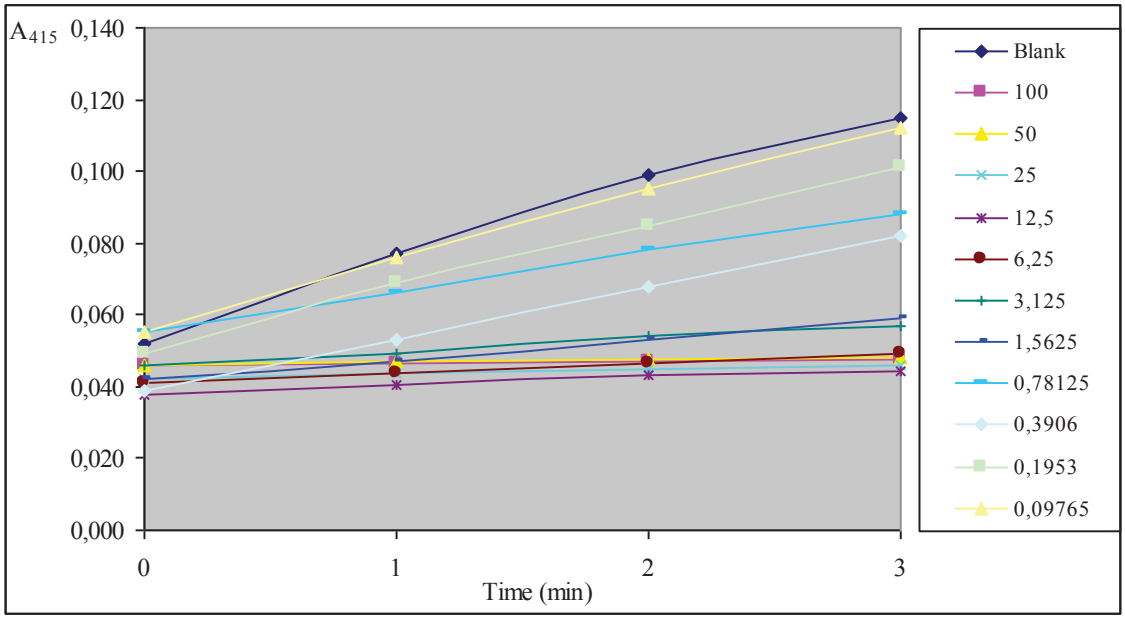

Figure 1. Linearity of standard curves using different concentrations of bovine Cu/Zn SOD (μU/ml)

On Figure 2 is showed standard curve of an SOD inhibition activity of pyrogallol autooxidation. The logarithmic function of standard curve was used for estimation of SOD activity in milk sample.

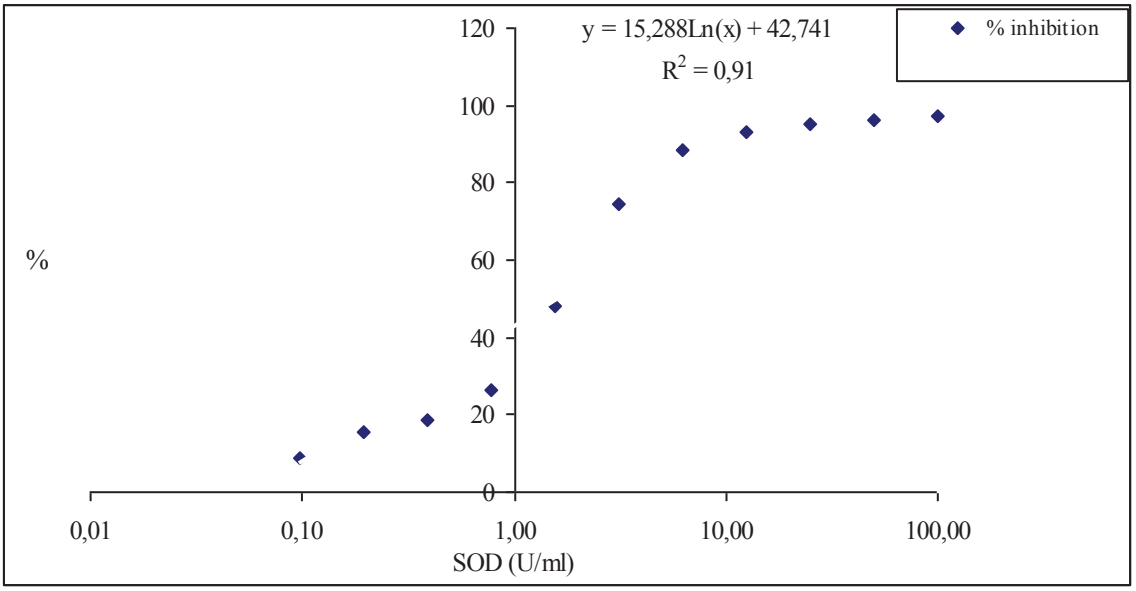

Figure 2. Representative standard curve using different concentrations of bovine Cu/Zn SOD. The regression equation was y=15.288Ln(x) + 42.741 with r = 0.91

Using milk samples, the linearity of the assay was established in a range between sample dilution 1:40 to 1:2.5 (Figure 3).

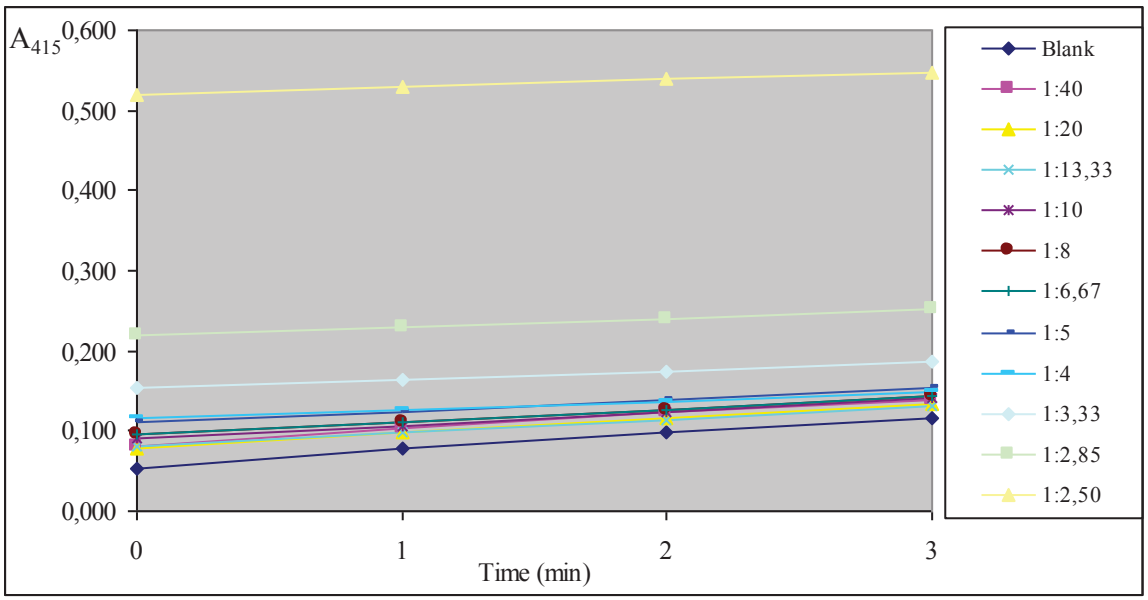

Figure 3. Evaluation of linear kinetic related to SOD activity using different volumes of milk serum

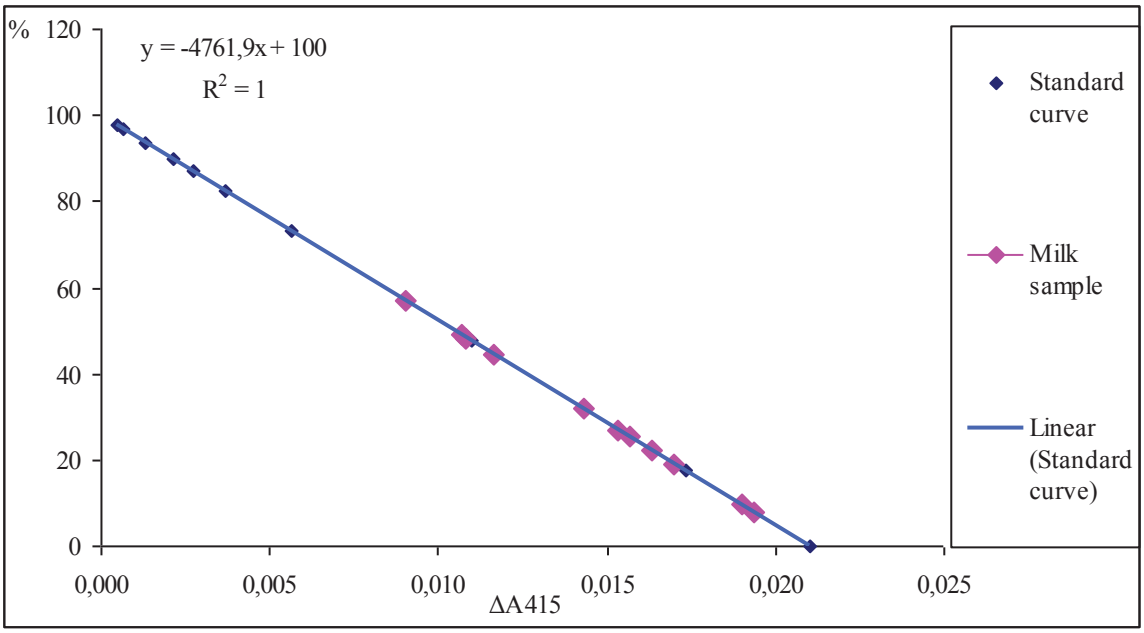

Figure 4. Net rate of absorbance change (standard curve vs. different dilutions of milk sample)

The limit of detection was defined as the concentration of bovine Cu/Zn SOD that yielded net rate of absorbance change different from the blank value measured by using H_2O and corresponds to the lowest standard concentration of 0.09765 U/mL SOD. The measured SOD activity in milk sample was in the range of standard curve which allow reliable measurements.

Figure 5 shows apparent rates of changes in absorbance at 340 nm as a measure of the oxidation of NADPH in the presence of milk, as a function of substrate concentration (GSH). The validity of this procedure is demonstrated by the constant relationship between decrease in absorbance at 340 nm per min and enzymatic NADPH consumption. Aimed to

get better linear kinetic in enzymatic NADPH oxidation, there was used different dilutions of milk sample and different concentration of GSH in reaction mixture. The linearity of the assay was established in a range between sample dilution 1:20 to 1:1000.

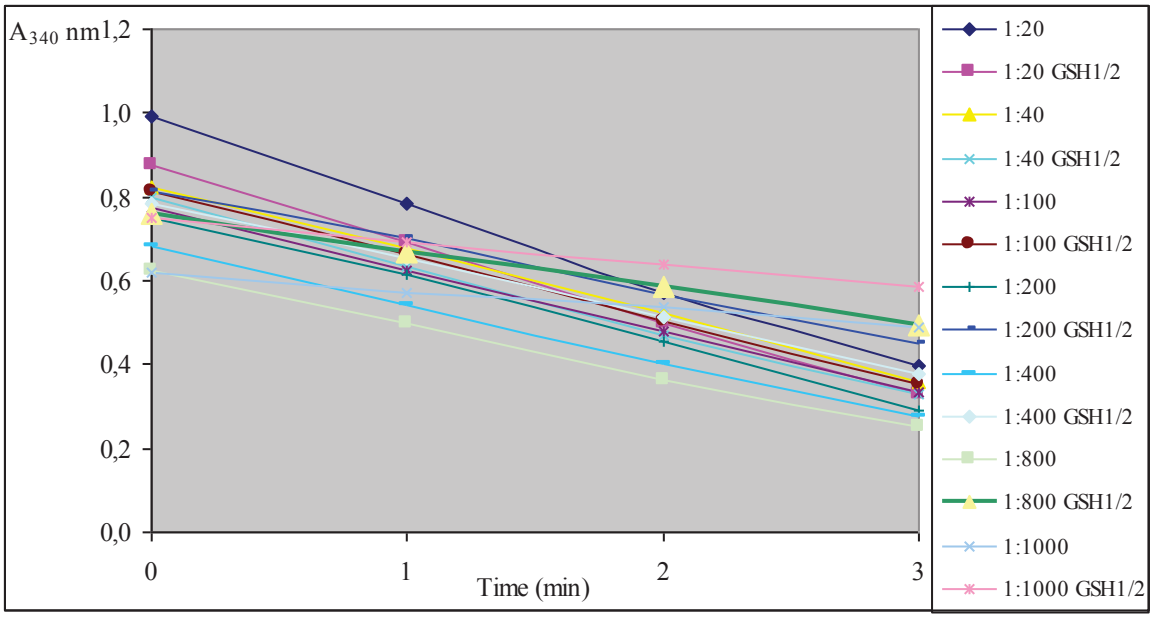

Figure 5. Evaluation of linear kinetic related to GPx activity using different volumes of milk serum

Discussion

Most procedures for determining SOD are based on the ability of the enzyme to inhibit oxygen-dependent reactions. Superoxide, produced by an in vitro reaction, reacts with the indicator giving absorbance at specific wavelength that increases with time. The correlation between the SOD concentration and the inhibition rates of the superoxide reaction with the indicator, allows to determine the enzyme activity. The inhibition of pyrogallol autoxidation brought about by SOD can be employed in a rapid and convenient method for the determination of the enzyme. After the above investigation we chose to use 0.2 mM pyrogallol in air-equilibrated 50 mM Tris-HCl buffer pH 8.5, containing 1 mM diethylenetriaminepentaacetic acid (DTPA).

Pyrogallol autoxides rapidly in alkaline aqueous solution, its autoxidation rate is measured from the linear increase in adsorbance at 415 nm for some minutes (usually from 1 to 3 minutes each assay). The increment of the band is linear within 7-10 minutes, then the colour turns green and finally, after a few hours, a yellow colour appears**.**

The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by the presence of SOD. As reported by Marklund and Marklund (1974), the rates of pyrogallol autoxidation were determined at pH 8.0 and 9.2. The rate of increase in absorbance at 420 nm per min was increased with increasing pH from 8.2-8.9. The autoxidation of pyrogallol was rapid at pH 9.2 but followed a non-linear pattern. By contrast, at pH 8.0 the autoxidation of pyrogallol was slow but followed a linear kinetics. We observed that better sensitivity and reproducibility were obtained when the pH of the assay mixture was kept at 8.5. At pH 7.9 the autoxidation is inhibited to 99% by Cu/Zn SOD, being the enzyme activity independent of pH in the range 5.5-9.5 (Gao et al., 1998).

The sensitivity to SOD decreases when the pH is increased, but still amounts to 93% at pH 9. At higher pH values there is a strong decrease in the sensitivity to SOD and at pH 10.6 the autoxidation is inhibited to only 15% by SOD (Violi et al., 1985).

Higher concentrations of pyrogallol decrease the sensitivity of assay (Gao et al., 1998). At 0.5 mM pyrogallol the sensitivity is decreased by a factor of 3.3. Iron, even in trace amounts, accelerates pyrogallol autoxidation. DTPA was found to prevent interference from Fe^{2+} (as well as from Cu^{2+} and Mn^{2+}) and was therefore chosen as chelator in the assay medium. In the absence of DTPA the autoxidation is faster than in its presence, and it tends to vary in rate and is less affected by superoxide dismutase. In the presence of DTPA the rate is independent of the concentration of the chelator, which indicates that the effect of DTPA is only due to its binding of traces of metal ions. Most measurements in the present report were performed in the presence of 1 mM DTPA. In the present method one unit corresponds to 100 ng bovine Cu/Zn SOD in a total volume of 1 ml; thus the method is about as sensitive as the method based on the reduction of cytochrome c by xanthine oxidase (Beyer and Fridovich, 1987). Using different amounts of the milk whey, the linearity was confirmed. This method is fast, cheap, simple, sensitive and requires common diagnostic tools as a commercial spectrophotometer featured by a visible light and very few amount of enzyme.

Glutathione peroxidase (GPx) activity has been measured through a coupled reaction with glutathione reductase (GR). In the assay, GPx reduce cumene hydroperoxide (cH_2O_2), and oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). The generated GSSG is reduced to GSH with through oxidation of NADPH to NADP+ by glutathione reductase (GR). The decrease of NADPH is proportionally to GPx activity in the reactions. The decrease of NADPH can be easily measured by absorbance at 340 nm.

Chen et al. (2000) found that GPx activity in added whey or milk was found to increase as buffer pH increased from 7.0 to 8.0, and a pH of 7.6 was selected to avoid a high water-blank. The same authors reported When the phosphate concentration was changed from 25 to 100 mM/L, GPx activity decreased and the blank increased, showing that lower buffer concentration favored GPx activity. When the assay was performed at 25^0C instead, the GPx activity in whey was 30% lower than at 37^0C. According them, the GPx activity in milk was only slightly higher than that in whey indicating that most of the GPx in milk was recovered in whey.

The oxidation of NADPH was dependent from the presence of GSH and increased by the addition of milk. The better sensitivity and reproducibility were obtained when assay was performed in presence of 1 mM GSH. Usually for milk assays a lower GSH concentration, a lower peroxide concentration and a more alkaline pH were used than in blood assays (Debski et al., 1987; Bhattacharya et al., 1988; Avissar et al., 1991). One reason for this is probably that erythrocytes contain mainly cellular GPx and milk mainly extracellular GPx (Bhattacharya et al., 1988; Avissar et al., 1991; Lindmark-Maensson and Akesson, 2000). An important difference between these two forms is that extracellular GPx has a lower rate constant for GSH than cellular GPx (Esworthy et al., 1993) and at cellular levels of GSH (millimolar) the eGPx reaction appears to be up to 10-fold slower than the cellular GPx reaction. Moreover, cellular GPx could not be saturated by GSH and that a high GSH concentration could inhibit GR, thus decreasing the overall reaction rate.

Furthermore, there was dependence between substrate addition and presence of milk sample in reaction mixture, where the oxidation of NADPH clearly increased following addition of cumene H_2O_2. Although the absorbance decreases during the assay

due to decreasing concentrations of NADPH, the negative rates have been converted to positive values throughout. Avissar et al. (1991) found the same GPx activity in milk using either H_2O_2 or tert-Butyl Hydroperoxide (t-BHPx) as substrate.

The presence of EDTA turned out to be critical for the apparent oxidation of NADPH by milk.

Contrary, some authors failed to detect any specific activity of GPx in bovine milk. Stagsted (2006) found GSH-independent NADPH oxidation in milk and GPx-catalysed t-BHPx - independent oxidation of GSH was observed. Author used 25 mM EDTA to allow optical measurements in concentrated milk samples. Although only 50% of the apparent turbidity could be eliminated with EDTA. Furthermore, they observed that concentrations of EDTA above 6 mM completely inhibited the apparent activity in milk. Therefore, we perform assay on milk whey, previously cleaned from turbidity and pH was adjusted to pH 7.6.

There are several reports on the activity of SOD and GPx in milk (Bhattacharya, et al., 1988; Debski et al., 1987; Avissar et al., 1991; Chen et al., 2000; Lindmark-Maensson and Akesson, 2000; Filipović et al., 2005; Kasapovic et al., 2005). According this reports, SOD activity in bovine milk was in the range from 0.92 to 3 U/ml. In raw cows' milk, extracellular form of GPx activity had demonstrated levels between 12 and 32 U/mL, and the activity has been found to be significantly correlated with selenium concentration (Przybylska et al., 2007). Debski et al. (1987) reported that GPx dependent peroxidase activity was approximately one-third of the total peroxidase activity and was found to be similar in human and bovine milk. However, some reports disagree regarding the measured activity of SOD and GPx in milk (Stagsted, 2006; Kovaceva et al., 2007).

Conclusıons

The present study describes an spectrofotometric kinetic protocols to measuring the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in milk whey. It has been shown that the linear kinetic observed in milk was depended from amount of whey sample and concentration of other compounds in reactive mixture. The both methods are reproducible and can be used for determination of antioxidant enzymes SOD and GPx in milk whey, suggesting that this antioxidative enzymes are of importance for milk stability and quality. SOD and GPx activity occurs in bovine milk, but their importance in this matrix is not well known.

References

Avissar N., Slemmon, J.R., Palmer, I.S. and Cohen, H.J. (1991). Partial sequence of human plasma glutathione peroxidase and immunologic identification of milk glutathione peroxidase as the plasma enzyme. Journal of Nutrition, 121, pg. 1243-1249.

Bandyopadhyay U., Das, D., Banerjee, K.R. (1999). Reactive oxygen species: oxidative damage and pathogenesis. Current Science 77, 658-665.

Beyer W.F.Jr. and Fridovich, I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem., 161, pg. 559-566.

Bhattacharya I.D., Picciano, M.F., Milner, J.A. (1988). Characteristics of human milk glutathione peroxidase. Biological Trace Element Research, 18, pg. 59–70.

Bordo D., Pesce, A., Bolognesi, M., Stroppolo, M.E., Falconi, M., Desideri, A. (2000). Cu,Zn superoxide dismutase in prokaryotes and eukaryotes. in: K. Wieghardt, R. Huber, T. Poulos A. Messerschmidt (Eds.) Handbook of Metalloproteins. John Wiley & Sons, London, UK; 2000.
Chen J., Lindmark-Mansson, H. and Akesson, B. (2000). Optimisation of a coupled enzymatic assay of glutathione peroxidase activity in bovine milk and whey. International Dairy Journal, 10, pg. 347-351.
Debski B., Picciano, M.F. and Milner, J.A. (1987). Selenium content and distribution of human, cow and goat milk. Journal of Nutrition, 117, pg. 1091-1097.
Esworthy R.S., Chu, F.F., Geiger, P., Girotti, A.W., Doroshow, J.H. (1993). Reactivity of plasma glutathione peroxidase with hydroperoxide substrates and glutathione. Archives of Biochemistry and Biophysics, 307, pg. 29–34.
Filipovic, D., Kasapovic, J., Pejic, S., Niciforovic, A., Pajovic, S.B. and Radojcic, M.B. (2005). Superoxide dismutase activity in various fractions of full bovine milk, Acta Aliment. 34 (3), pg. 219-226.
Gao R., Yuan, Z., Zhao, Z., Gao, X. (1998). Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. Bioelectrochemistry and Bioenergetics 45, pg. 41-45.
Halliwell B. (1987). Oxidants and human disease:some new concepts. Federation of American Societes for Eaperimental Biolog, 1, pg. 358-366.
Halliwell B. and Gutteridege, J.M.C. (2007). Free Radicals in Biology and Medicine, 4th ed. Oxford University Press.
Kasapovic J., Pejice, S, Mladenovice, M., Radlovice, N., Pajovice, S.B. (2005). Superoxide dismutase activity in colostrum, transitional and mature human milk. Turkish J Pediatr 47, pg. 343-347.
Kovaceva J., Platenik, J., Vejrazka, M., Stipek, S., Ardan, T., Cejka, C., Midelfart, A., Cejkova J. (2007). Differences in activities of antioxidant superoxide dismutase, glutathione peroxidase and prooxidant xanthine oxidoreductase/xanthine oxidase in the normal corneal epithelium of various mammalia, Physiol. Res. 56, pg. 105-112.
Lindmark-Mansson, H. and Akesson, B. (2000). Antioxidative factors in milk. British Journal of Nutrition, 84, pg. 103-110.
Marklund, S. and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, pg. 469-474.
Nielsen J.H., Hald, G., Kjeldsen, L., Andersen, H.J. and Ostdal, H. (2001). Oxidation of ascorbate in raw milk induced by enzymes and transition metals. Journal of Agricultural and Food Chemistry, 49, pg. 2998-3003.
Paglia D.E. and Valentine, W.N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, pg. 158-169.
Przybylska J., Albera, E. and Kankofer, M. (2007). Antioxidants in Bovine ColostrumReprod Dom Anim 42, pg. 402-409.
Stagsted J. (2006). Absence of both glutathione peroxidase activity and glutathione in bovine milk International Dairy Journal 16, pg. 662-668.
Violi F., Iuliano, L., Alessandri, C., Ghiselli, A. and Balsanoa, F. (1985). Simple method for evaluating platelet superoxide dismutase. Scand J Clin Lab Invest 1985; 45: pg. 713-716.