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Preliminary studies on the activity of superoxide dismutase from different meat extracts

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Abstract

The valuable antioxidant activity of superoxide dismutase (SOD) involved in protective mechanisms against ROS (reactive oxygen species) was monitored. SOD activity was evaluated in two crude meat extracts by measuring the ability of SOD to inhibit the autoxidation of pyrogallol, with the aim to further understand its thermal and athermal stability.

The activity of superoxide dismutase extracted from *Longissimus dorsi* muscle of pork and pork kidney extracts was measured in the presence of phosphate buffer with 1 mM EDTA, pH 8.2, using 0,15 mM pyrogallol and 10 mM HCl as substrate. SOD activity in kidney and *Longissimus dorsi* muscle extracts was spectrophotometrically assessed.

SOD extracted from pork kidney displayed higher activity values than the one from *Longissimus dorsi* muscle, thus it was further selected for the inactivation studies. This preliminary study of SOD activity demonstrated that kidney extracts are richer in SOD and could represent a better choice for performing thermal and athermal inactivation.

Keywords: enzyme activity, superoxide dismutase, antioxidant activity.

1. Introduction

Meat lipids and the fatty tissue undergo important chemical and biochemical changes during refrigeration and freezing processes, influenced by salts concentration and by the equilibrium between the pro-oxidant and antioxidant systems. The complex oxidative mechanisms, which are involved in the muscle, lead to the formation of reactive oxygen species and various oxidizing compounds. The most representative oxidative species are hydroxyl radicals, peroxyl radicals, superoxide anion, H_2O_2 and nitric oxide. Usually, the reactive oxygen species (ROS) can interact with both lipids and proteins [1-2]. The antioxidant enzyme superoxide dismutase (SOD) was for the first time studied in 1969 by Dr. McCord, and Professor Irwing Fridovich 3]. Superoxide dismutase plays an important role in preventing the damage caused by the superoxide anion radical [4-6]. The fundamental property of superoxide radical is to reduce the hydrogen peroxide [7]. SOD is a metal protein, with various cofactors (Cu, Zn, Mn, Fe) and has essential roles, such as: to control the level of free radicals in the body [6-8] and to prevent chronic diseases [2, 9-11]. Also, this enzyme is a tetrameric glycoprotein which catalyses the removal of superoxide, generating hydrogen peroxide as the final product of dismutation [6, 12]. There are several types of SOD isoenzyme and while CuZn is found in the cytoplasm, nucleus and plasma, Mn SOD is localized in mitochondria [6-7, 10, 12-13].

The objective of the current research was to evaluate the total SOD activity in two crude meat extracts by measuring the ability of SOD to inhibit the autoxidation of pyrogallol, with the aim to further understand its thermal and athermal stability.

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2. Materials and Methods

The pork muscle extract was obtained following certain steps: cutting, chopping and homogenization 1:2 with phosphate buffer (PB) pH 8.2, extraction during one hour at 4°C, centrifugation for 10 minutes at 3500 rpm, supernatant separation, filtration and freezing the filtered sample.

Fresh *Longissimus dorsi* (LD) pork muscle (from one day slaughtered animal) was cut into small pieces and then chopped using the Kitchen Aid KSM 150 kitchen robot to improve the extraction yield. The minced meat was mixed 1:2 with phosphate buffer pH 8.2 and kept under refrigeration conditions to obtain the extract. After homogenization of the meat and the phosphate buffer, the mixture was stored for one hour at 4°C for extraction. The extract was centrifuged at 3500 rpm for 10 min and the supernatant was collected. Then, the supernatant was filtered under vacuum to obtain a clear extract.

The pork kidneys without strikes or cuts was selected to avoid the presence of intense oxidative processes. The kidney was cut into pieces and the fat inside was removed.

The kidney's chopping was performed with the Kitchen Aid KSM 150. The homogenate was obtained in the same way as in the pork muscle. The extraction was carried out over one hour at 4°CThe centrifugation of the homogenate was performed in a Refrigerated Centrifuge TGL-16M at 9000 x g [14] for 20 min at 4°C and a clear, intense red supernatant was obtained. The filtration was carried out with the vacuum filtration.

In order to determine the influence of storage temperature on SOD activity, for both extracts, one part of the clear extract was frozen at -18°C, and the other was stored under refrigeration conditions for further analysis.

The method for determination of the activity of superoxide dismutase was optimized using various samples extracts (*Longissimus dorsi* muscle of pork and pork kidney).The SOD activity was evaluated using pyrogallol, which is a substrate of the enzyme and phosphate buffer (monosodium and disodium phosphate), providing a constant pH to the sample. Therefore, the hydrochloric acid was used to obtain an increased stability of the pyrogallol, from 3 to 12 hours. EDTA has a significant role and interfere with metal chelation in order to release the enzyme from the food matrix and sodium hydroxide to adjust the pH.

Total SOD activity was determined using the supernatant, based on the inhibition of pyrogallol according to the method of Marklund [15-16]. SOD activities of both *Longissimus dorsi* muscle extract and pork kidney were measured at 420 [17-20] and 320 nm, respectively, using T80+ UV/VIS Spectrometer.

3. Results and Discussions

In order to determine the optimum reaction conditions of SOD, the enzyme extract from the pork kidney was considered as a reference sample (in the absence of pure enzyme), in which the SOD activity was found to be higher than in the LD porcine muscle extract. The SOD enzyme activity was measured spectrophotometrically at 320 nm varying the extract concentration. The autoxidation activity of pyrogallol was measured at different SOD concentrations from kidney extract as function of the volume of the extract (Figure 1) added.

The values depicted in Figure 1, show an inverse relation of proportionality between the SOD concentration in the sample and the antioxidant activity of pyrogallol. However, when it was used a double amount of extract or enzyme, the autoxidation activity of pyrogallol shows a sharp decrease, reaching to 0.009 relative units of activity compared to the activity of 0.0125 recorded at a SOD concentration of 1.33. After a 10 fold increase in the extract concentration, the pyrogallol autoxidation activity exhibits an almost constant values, the absorbance measured being 0.0005 at a SOD concentration of 10 and 0.0004 at a concentration of 13.3300, respectively. It was considered that with the extract dilution, the SOD concentration will be reduced proportionally to the applied dilution. So, the increase of the pyrogallol autoxidation activity is due to the dilutions applied to the pork kidney extract (Figure 1). Thus, with the 1:15 dilution corresponding to the SOD concentration of 0.066, the amount of enzyme in the extract is reduced, and the autoxidation activity of the pyrogallol increases, reaching a value of 0.0161. Therefore, the pyrogallol autoxidation activity is 40 times higher than that of a 200 fold concentrated enzyme assay, corresponding to the SOD concentration of 13.33.

The investigation of the SOD activity was performed through the analysis of certain physico-

chemical parameters, such as the influence of phosphate buffer (PB), pH and substrate concentration, respectively. The influence of *p*H on SOD activity was evaluated using different values of PB pH (7; 7.4; 7.6; 8; 8.2 and 9). It has been observed that at pH 9, the activity of SOD activity increases significantly, meanwhile at pH 7, the activity decreased due to exceeding the optimal pH range. The optimal pH value was 8.2 where the enzyme activity was the highest, similarly to Serra and Falconi [8, 21-22]. EDTA was used in phosphate buffer to assure the chelation capacity of some metal ions (Ca2+, Fe3+, Mn2+) from SOD enzyme.

The maximum activity of SOD enzyme was investigated by varying the EDTA concentration (0.1, 0.5, 1, 1.5 and 2 mM) added in the PB solution.

The most suitable system for our analysis was the phosphate buffer with pH 8.2 and 1 mM EDTA. This concentration of EDTA was considered optimal to support the desired chelation effect without altering the initial working conditions but only in order to increase the activity of superoxide dismutase according to the literature [10, 15-16]. In Figure 2, the SOD activity variation from kidney extract was shown as function of extract sample when it is added 1 mM EDTA to the phosphate buffer.

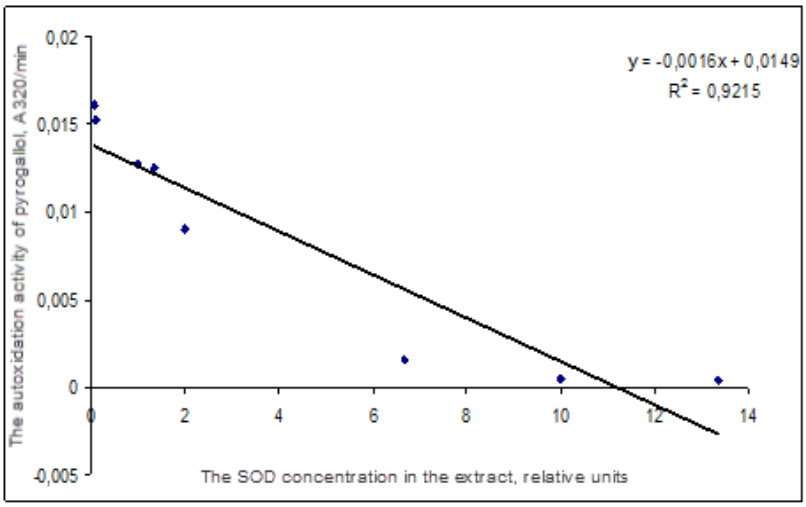

Figure 1. Autoxidation activity of pyrogallol in the presence of the SOD enzyme from pork kidney as function of the extract concentration

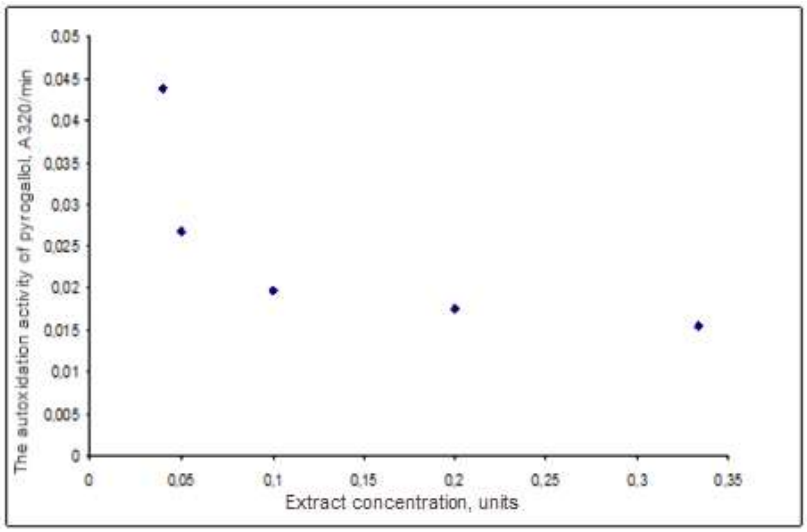

Figure 2. SOD activity from pork kidney extract as function of the extract sample amount containing 1 mM EDTA in phosphate buffer (PB)

According to the Figure 2, the absorbance values indicate an increase in the pyrogallol autoxidation activity depending on the dilution of the pork kidney extract. The added EDTA increases with 78% the SOD protection capacity of the extract at a concentration of 0.1 units on the pyrogallol autoxidation activity compared to the sample where no EDTA has been added to the phosphate buffer in the sample. Next, the influence of the storage temperature of the extract on SOD activity in order to choose the optimal storage method between refrigeration and freezing was determined.

In Figure 3, it can be seen that the SOD enzyme in the first day of pork kidney extract refrigeration showed a slightly lower activity of 0.0006 units compared to that of the fresh extract which had an activity of 0.0007 units ($p<0.05$). This proved that, the enzyme is unstable under refrigeration conditions.

From Figure 3, it can be observed a 57% decrease of SOD activity in the refrigerated pork kidney extract over a 5 day period compared to the enzyme activity of 0.0007 relative units in the freshly filtered extract. Under freezing conditions at -18 °C, the SOD activity in the defrosted extract has the same value of 0.0006 relative units after 5 days of freezing. So, the selected storage method was the freezing process at -18 °C by filtering the pork kidney extract. From the freeze dried kidney extract, a dilution of 1:3 was prepared and the activity of SOD in this diluted extract was evaluated at different concentrations of the substrate (pyrogallol): 0.01, 0.05, 0.1, 0.3 and 0.5 M. In order to slow the pyrogallol autoxidation, different concentrations of HCl (1, 5, 10, 15 and 20 mM) were used. A10 mM HCl concentration was added to pyrogallol 0.15 M to assure the SOD enzyme substrate stability, without to reduce excessively the activity of superoxide dismutase in the extract.

All these experimental conditions were used to determine the SOD enzyme behaviour from pork kidney, whereas, in the case of the LD pork muscle, a 1:5 dilution of the initial extract (it was used Figure 4).

A significant increase of SOD activity was recorded in case of 1:5 dilution of LD pork muscle extract due to the pyrogallol concentration increment. The enzyme activity was around 0 at 0.001 M pyrogallol and 0.0024 (maximum activity) at 0.5 M pyrogallol, respectively.

The highest variation of the pyrogallol concentration was noticed between 0.01 M and 0.05 M, when the SOD activity exhibited a significant increase from 0.0004 to 0.0019, almost 5 times higher.

Also, it the SOD activity of LD pork muscle was investigated as a function of the concentration of the extract. The variation of the pyrogallol autoxidation under the influence of SOD enzyme from LD pork muscle extract is shown in Figure 5.

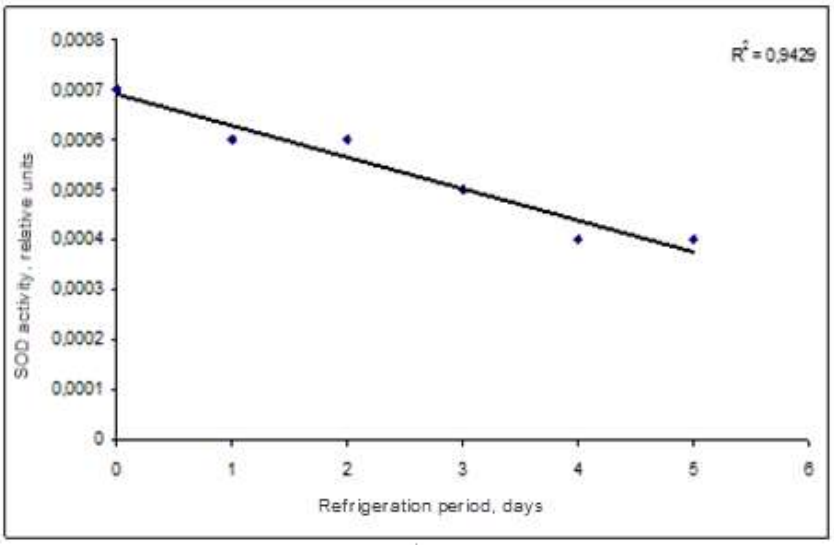

Figure 3. The influence of the refrigeration period on the SOD enzyme activity

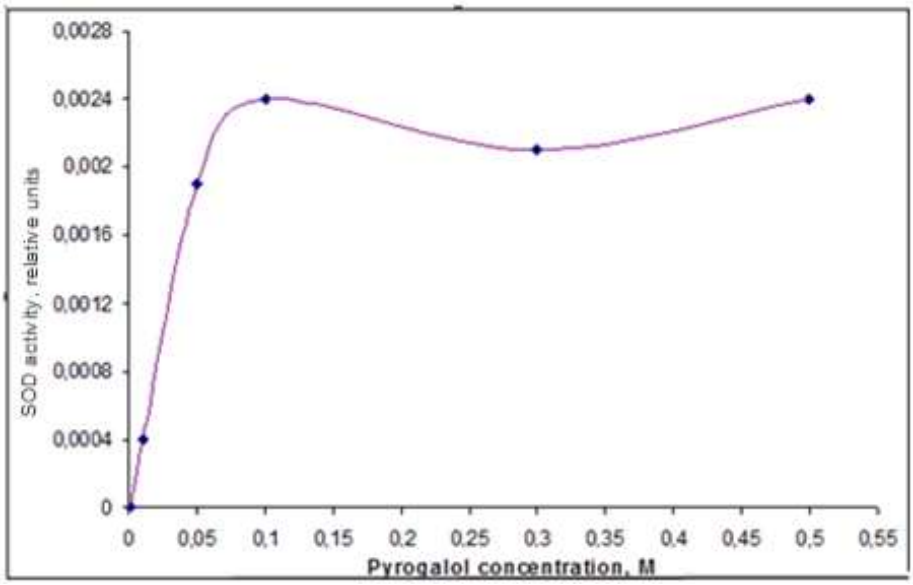

Figure 4. SOD activity variation of the LD pork muscle as function of the enzyme concentration (1:5 dilution) in relation to the pyrogallol concentration

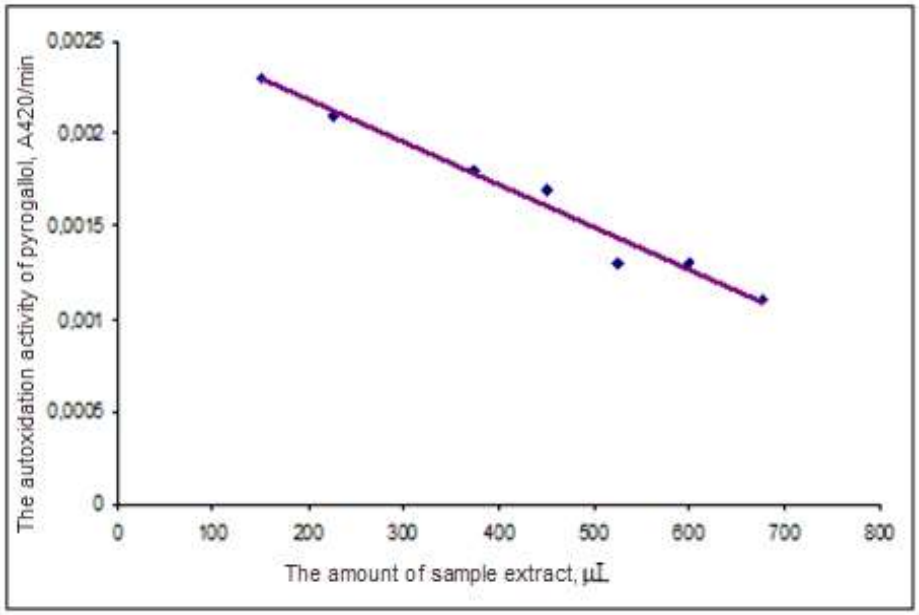

Figure 5. The variation of pyrogallol autoxidation activity under the influence of SOD from LD pork muscle extract, depending on the amount of sample extract

A decrease of the absorbance was observed, inversely correlated with the increase of the extract volume from 150 µL to 470 µL, which shows a greater protection against to the oxidation, provided by the increase of the SOD extract concentration. The enzyme has a protective role against to the pyrogallol autoxidation, for example in the case of 675 µL extract, the pyrogallol exhibits a lower autoxidation activity of 0.0011 relative units. The most obvious reduction of the autoxidation was observed by comparing these results in the first sample (1:5 dilution), in which the extract quantity was 5 times smaller and the autoxidation activity that was 2 times smaller.

Various analysis were developed to obtain an optimal enzyme/substrate ratio in the sample. In the case of substrate concentration variation, a linear increase of the SOD activity from LD pork muscle extract was observed, depending on the substrate concentration (pyrogallol). So, the enzyme behaviour was explained by the higher protection of SOD on pyrogallol oxidation.

4. Conclusion

The antioxidant SOD activity was monitored according to the protection against pyrogallol oxidation. SOD activity was determined by spectrophotometric analysis.

The decrease of the autoxidative activity of pyrogallol was observed with increasing concentration of the sample extract.

The optimum SOD activity of the pork kidney extract was noticed in the presence of phosphate buffer with 1mM EDTA pH 8.2, using 0,15 mM pyrogallol and 10 mM HCl as substrate. The cumulative effect of the concentrations of these reagents implies identical reaction conditions every time and the results could be repeated and replicated.

The activity was not affected by freezing and was reduced by refrigeration. After 5 days of refrigeration the activity decreased with 57 % compared to the fresh extract.

This preliminary study will allow to continue the researches concerning the SOD inactivation kinetics under different pressure and temperature conditions.

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Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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