FINAL REPORT
September 17, 2012

"LIVESTOCK FEED PRESERVATIVES BASED ON ANTIOXIDANT ENZYMES EXTRACTED FROM ANIMAL BLOOD"

Principal Investigator(s):

Vladimir Reukov, PhD, Research Assistant Professor
Department of Bioengineering
301 Rhodes Hall
Clemson University
Clemson, SC 29634
Phone: 864-643-7937
Fax: 864-656-6644
E-mail: reukov@clemson.edu

Alexey Vertegel, PhD, Associate Professor
Department of Bioengineering
301 Rhodes Hall
Clemson University
Clemson, SC 29634
Phone: 864-656-0801
Fax: 864-656-6644
E-mail: vertege@clemson.edu

Date Submitted: September 17, 2012

Start Date: July 1, 2010

Duration of Project: 36 months

Lay Summary:

This project is directed on extraction of antioxidative enzymes from animal blood and their use as feed preservatives. Oxidation of oils and lipids in animal feeds leads to deterioration in taste, color and texture. The industry seeks natural solutions to extend the shelf life of the products while maintaining product freshness and quality. Currently-used antioxidants such as tocopherols and phenolic (e.g. *butylated hydroxyanisole* (BHA)) and nitrogen (e.g. *ethoxyquin*) compounds, as well as vitamins E and C are expensive and do not always fulfill their intended roles. The enzyme **superoxide dismutase (SOD)** derived from animal blood, evinces excellent anti-oxidative properties, being an **effective free-radical scavenger**. Here, we propose to establish a simple method to produce SOD concentrate from animal blood and study its applicability and stability as an antioxidant feed supplement.

Objective (s):

The objectives of this research are:

- To develop simple method for production of purified enzyme concentrate from animal blood.
- To study enzyme concentrate activity against free radicals and optimize the extraction method so to achieve maximum activity
- To maximize the shelf life of the enzyme concentrate using the appropriate additives to the storage solution
- To test antioxidant activity of our enzyme concentrate and compare its performance to that of currently used antioxidants (Pet Ox (BHA + BHT), Nature Ox);
- To study enzyme concentrate performance as food preservative after accelerated shelf life study (50°C, 2 weeks) and compare it to currently used antioxidants;
- To estimate costs, associated with extraction of SOD from animal blood.

Project Overview:

Oxidation of oils and lipids in animal feeds leads to deterioration in taste, color and texture. The industry seeks natural solutions to extend the shelf life of the products while maintaining product freshness and quality. Currently used antioxidants such as vitamins C and E or tocopherol are expensive and do not always fulfill its role. On the other hand enzyme **superoxide dismutase derived (SOD)** is evincing excellent anti-oxidative properties being an **effective free-radical scavenger**. Superoxide dismutase is several thousand times more efficient in eliminating free radicals than conventional antioxidants such as vitamin C. Although most of the anti-oxidative enzyme application principles were patented in the early 1970s and were demonstrated as efficient in various model systems, it had not been widely used in food industry. One possible reason for this is poor stability of the cheapest yeast-derived SOD. However, more expensive SOD derived from bovine blood is more stable and could be further stabilized using fatty acids with effective shelf life of 3 years at room temperature. Here, we propose to establish a simple method to produce SOD concentrate from bovine blood and study its applicability as an antioxidant feed supplement. Current method of SOD production from animal blood for research and medical purposes consists of the following steps: erythrocytes extraction by centrifugation, cell lysis, non-aqueous solvent

extraction of SOD, purification by column chromatography. All the steps except for the last one are very simple and cost-effective and can be performed by a non-specialist using simple equipment. The last step – enzyme purification by chromatography – is the main contribution to the high cost while being essential only for R&D purposes but could possibly be omitted for applications as an animal feed additive.

Introduction:

1. Review of Related Research/Literature Review

There is currently much interest in substituting synthetic food preservatives and synthetic antioxidants for substances that can be marketed as natural. During the last two decades, many antioxidative protein hydrolysates and peptides have been discovered or developed. These natural antioxidants are being evaluated for potential commercial applications to preserve the quality of fresh and processed food. Currently herb-derived SOD is demanded human diet supplement which is widely spread on the market (S.O.D. 2000 Plus ™ by Solaray, GliSODin™ by Now), as a key cellular antioxidant that is instrumental in neutralizing the highly potent superoxide radical, thereby constituting an important system for the protection of cells and tissues against degeneration by free radicals. Bovine erythrocyte SOD has been extensively studied and it's found to be identical to the enzyme from human erythrocytes and from beef heart. Recent animal diet studies showed that levels of SOD activity in grain-fed animals was much lower than in pasture-fed which leads to decrease of meat quality and oxidative stability. In the year 2008 clinical trial in France has been completed on use of the Superoxide Dismutase (SOD) as Antioxidant Treatment OF Age Related Macular Degeneration (ARMD). On cellular level, SOD outcompetes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. The superoxide anion radical (O_2^-) spontaneously dismutes to O_2 and hydrogen peroxide (H_2O_2) quite rapidly (~10^5 $M^{-1}s^{-1}$ at pH 7). SOD is necessary because superoxide reacts with sensitive and critical cellular targets. Moreover, superoxide dismutase has the largest k_{cat} (reaction rate with its substrate) of any known enzyme (~7×10^9 $M^{-1}s^{-1}$),[10] this reaction being only limited by the frequency of collision between itself and superoxide. These unique product can be used to inhibit oxidative deterioration in foods and to prevent certain adverse health conditions in humans and also if fed to animals could significantly protect lipids in meat from oxidation.

2. Experimental Procedures

During the first year of the project, we modified currently used procedure for SOD extraction from animal blood. Erythrocytes were collected from fresh bovine or porcine blood (1000 ml) containing some anticoagulating agent as heparin (10 units/ml) by centrifugation (600 g, 10 rain). The packed cells were lysed by addition of 2 volumes of cold distilled water and PMSF(phenylmethylsulfonyl fluoride, lysing agent) was added (final concentration, 10 μM). Solid ammonium sulfate was added with stirring to the hemolyzed sample to make a 90% saturated solution (662 g/l.1). The following steps were performed at room temperature. The ammonium sulfate solution was adjusted to pH 5.0 with acetic acid, and an equal volume of

methanol was added slowly with stirring. The resulting mixture was vigorously stirred for 15 min. Centrifugation (3000 g, 10 min) of the mixture will result in a separation of liquid and solid phases; the former contained enriched SOD activity and the latter contained denatured hemoglobin, other proteins and insoluble ammonium sulfate. Methanol was removed by centrifugation using Microsep membrane and finally SOD was concentrated to 50 ml.

To study enzyme concentrate activity against free radicals SOD Assay Kit – WST (Cat. No. 19160, Fluka, St. Louis, MO) was used. This assay employs a water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt), which produces a soluble formazan dye upon reduction by superoxide. The assay kit was used according to the instructions provided by the supplier. SOD solutions in PBS with known activities of 0.05-3780 U/mL were used as the standards. To study effect of synergy on another anti-oxidative enzyme – Catalase, which usually derived from bovine or porcine liver, we measured Catalase activity using standard biological activity assay.

The concentration of accompanying enzymes and level of enzymatic activity was the key data for determination of prospective application. And high SOD activity will be useful for anti-oxidative food application – to inhibit degradation of oxidizable substances. Another possible application – is incorporation of these anti-oxidants in food packaging materials, like polymer films or wraps.

During project progressing, we modified initial SOD extraction procedure to maximize the extraction yield and decrease the production costs.

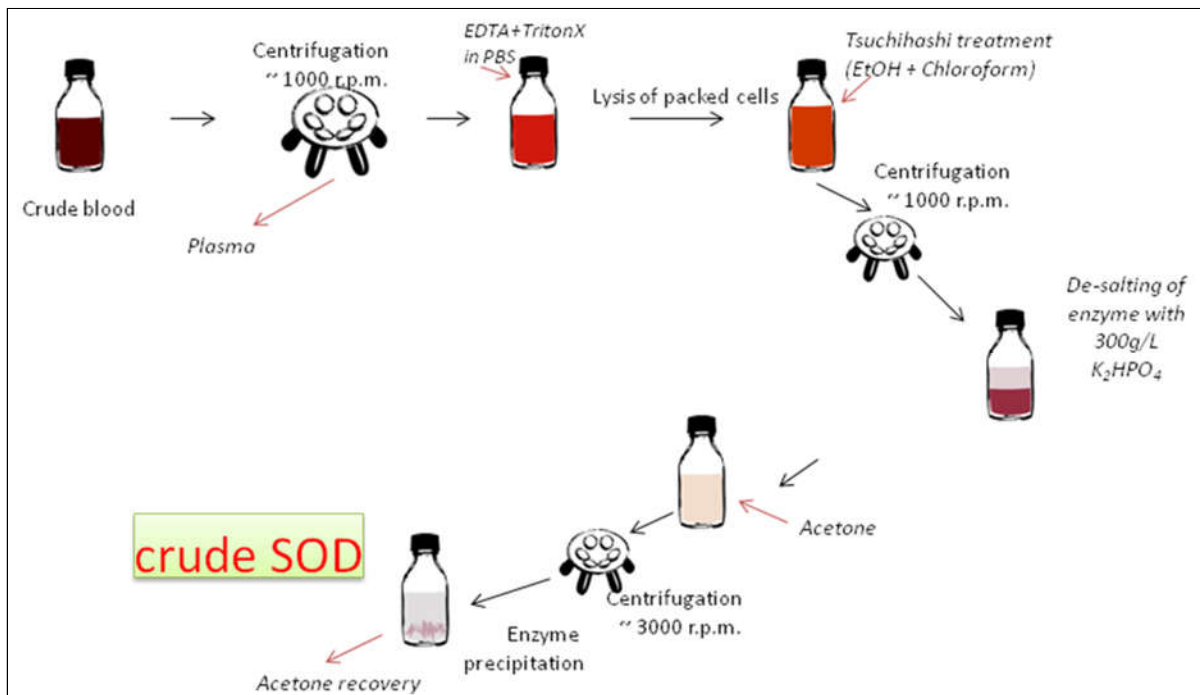

Fig.1. SOD extraction protocol

Current detailed procedure for enzyme extraction is the following:

1. All the porcine and chicken blood samples were collected from Wilson's meat processing, Seneca and Fieldale Farms, Georgia respectively; and stored at 4ºC upon receipt. Long term storage was done at-20ºC.The entire procedure was carried out at 4ºC unless otherwise mentioned.
2. To begin with, the samples were centrifuged at 500-1000g to obtain a pellet of red blood cells (RBCs) and the plasma was removed. This was followed by washing with Dulbecco's 1X PBS, to effectively remove any plasma and other clotting factors.
3. The packed RBC's were then lysed in a lysing solution, which was same as the existing blood volume. It consisted of 1% Tween20 and 1% TritonX, EDTA 0.1mM (0.037g/L, Mallinckrodt Baker Inc, Paris Kentucky, USA).
4. The lysis was carried out for 25 mins under vigorous shaking and the resulting hemolysate was then subjected to the Tsuchihashi treatment [consisting of chloroform (HPLC Grade, EMD, USA) and ethanol (anhydrous, denatured, ACS, 94-96%, Alfa Aesar, MO, USA) added in a ratio of 0.25:0.15 of the hemolysate volume].
5. Vigorous stirring of the treated hemolysate was done for 25 mins resulting in a brick red solution. Subsequent centrifugation at 4000g for 15 mins resulted in haemoglobin precipitating and the chloroform phase separating out. The relatively clear supernatant (ethanol phase) was collected from the top.
6. K_2HPO_4 (Sigma Life Sciences, St. Louis MO,USA) (300g/L) was added; followed by centrifugation at 5000g for 15 mins to precipitate any additional extraneous proteins and remove left over chloroform(discard bottom liquid into chemical waste jar).
7. The pale yellow supernatant was collected and acetone (ACS,>99.5%, Sigma Life Sciences, St. Louis MO, USA) stored at -20ºC was added to the solution in a ratio of 2:1 and centrifuged at 5000g for 15 mins to ensure precipitation of the desired protein of interest (precipitate appeared creamish in color).
8. The supernatant was removed and the protein was washed and centrifuged at 5000g twice in 1X Dulbecco's PBS.
9. The purified protein solution was used in the SOD assay to quantify the amount of extracted enzyme.
10. Solution was frozen and freeze-dried, resulting in fluffy pink protein powder.

The following figures below shows the different stages of protein extraction:

Fig. 2. SOD extraction process captured at different stages: (1) RBC packed with centrifuge; (2) Lysis buffer added; (3) Tsuchihashi reagent added; (4) After centrifuging collecting top part – transparent organic/water mixture; (5) Adding K_2HPO_4 upto 300g/L or more if needed; (6) Adding acetone from the freezer – precipitating SOD from solution

Results & Discussion:

Study of the lysate activity was performed using SOD Assay Kit as described earlier. SOD solutions in PBS with known activities of 0.01-2000 U/mL were used as the standards. The major results of completed SOD activity assay are shown in Fig.3. The analyzed activity of enzyme concentrates from chicken and porcine blood was measured as ~142 and ~176 units per mL of initial blood sample, correspondingly. The antioxidative activity of chicken blood hemolysate was measured as ~240 U/mL, resulting in ~60-70% yield of enzyme. Results, acquired using modified procedure, are very promising and suggest that the proposed project has immense potential.

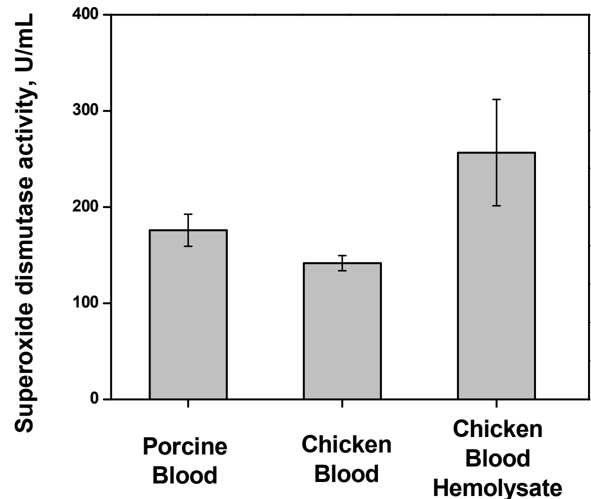

Fig.3. Results of SOD activity assay

The final step of the modified procedure – freeze-drying of concentrated enzyme solution were performed for 2 samples. Fig.4 shows the tube with freeze-dried sample featuring pinkish colour and fluffy structure of the obtained powder. Unfortunately the activity of resuspended powder was lower than expected. The reason for such low activity is that prior the freeze-drying the proteins were dissolved in distilled water, not containing necessary stabilizers (usually, potassium phosphate).

During the first year of the project we performed an analysis of available scientific literature. Further, pilot experiments of blood separation and SOD extraction were carried out and their activity were characterized. Later, based on an adjusted procedure SOD was separated from animal blood and the composition of final concentrate and enzymatic activity was studied.

Fig.4. Image of freeze-dried SOD concentrate.

During the first year of the project, we managed to optimize extraction protocol for the antioxidative enzymes concentrate from animal (porcine, chicken) blood. We also improve the extraction yield up to almost 70-90% of initial antioxidative activity, and increase the final concentrate activity to ~2400U/mL. We've performed enzyme extraction from chicken and poultry blood collected at Fieldale Poultry plant and a local slaughter-house, in similar conditions to mimic rendering collection procedure, except for addition of anti-coagulating agent *ethylenediaminetetraacetic acid* (EDTA). Samples were collected from the Fieldale Poultry plant (Cornelia, Ga) – chicken blood, and a local slaughter-house – porcine and bovine blood. Previously, during pilot studies the blood samples were sent to us by overnight delivery and didn't contain any preservatives. Hence, blood clotting and low SOD extraction yield was observed. To prevent blood coagulation and ensure complete enzyme

extraction, anticoagulant EDTA was added. Bulk price for this commonly used anticoagulant is about $1/lb, and this amount is suitable to preserve more than 100 liters of animal blood.

During the second year of the project, we have focused on preserving enzyme activity during the freeze-drying/lyophilization procedure. Initially we found that standard technique usually applied for isolation of proteins from solutions didn't work for our enzyme concentrate.

The standard protocol consists of following steps – first, the cryoprotector (typically polysaccharides like sucrose) was added to the enzyme extract, following by overnight freezing of the solution at -80°C.The frozen sample was then moved to a freeze-dryer, wherein its core temperature of -50^{0}C and a pressure ≤ 133mBar resulted in sublimation of water, leaving behind the lyophilized protein. After several attempts, using 1% of different cryoprotectants as stabilizers; (Trehalose, Sucrose, Dextrose and no additives) and various freezing and freeze drying protocols, we managed to find conditions at which the freeze-dried enzyme retained the maximum activity – the winning combination, which retained ~80% of the initial activity, was addition of 1% Trehalose and sufficient reduction of freezing time – from overnight at -80°C to 3hrs@-80°C. The results comparing different cryoprotectants are shown in Fig.1.

Our next goal was to study enzyme concentrate SOD activity after accelerated shelf life study. In order to complete this goal, the freeze dried samples obtained from the previous experiment, stabilized using the above mentioned cryoprotectants, were incubated at 60°C (3 weeks at this temperature is equivalent to 1 year at room temperature) for four days. Thereafter, the samples were removed and allowed to reach room temperature, before analyzing their SOD activity. Our initial findings were somewhat discouraging – all samples completely lost their

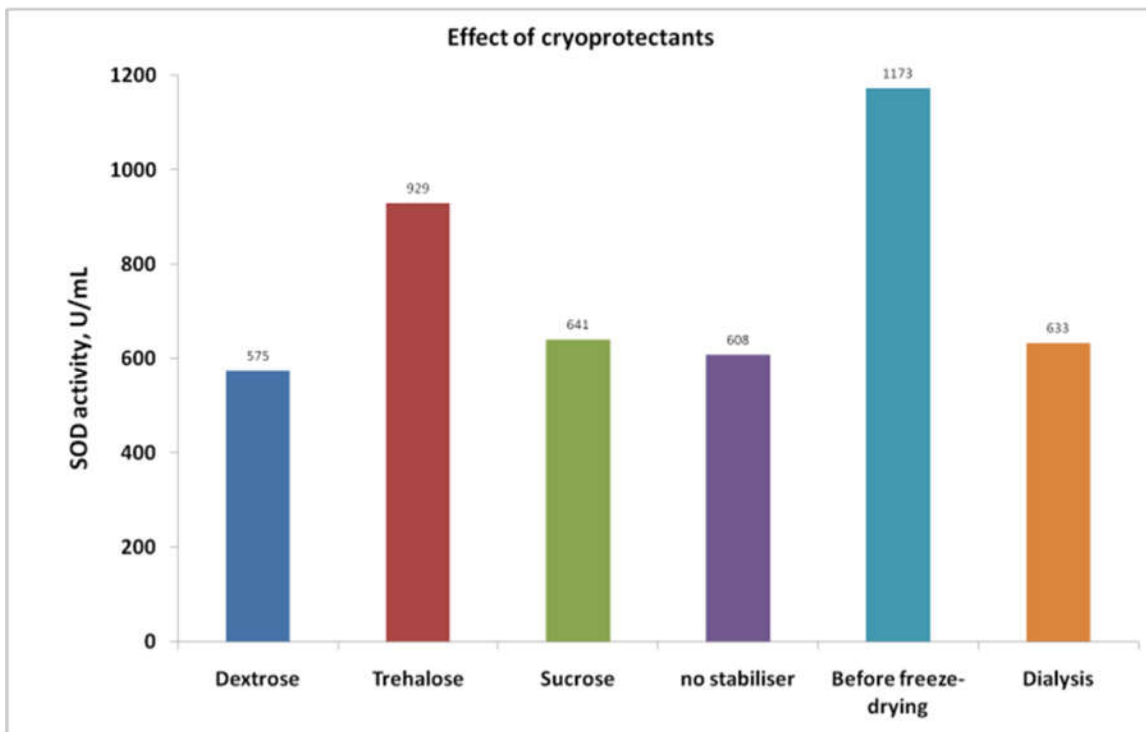

Figure 5. Effect of addition of cryopreservants on SOD activity after freeze-drying.

activity after four days. This result was particularly disappointing because pure SOD is known to

be a heat stable enzyme and should be stable even at this temperature. The possible reasons for loss of activity, we hypothesized, was due to a combination of factors like lack of purity of our extract, conformational changes to the protein structure exacerbated due to the drastic temperature changes (from –80°C during freezing to 60°C during the accelerated aging study) and possibly the lack of the right stabilizer concentration that could prevent such deleterious effects. Thus we decided to decrease the accelerated shelf-life study temperature to 50°C (1 week

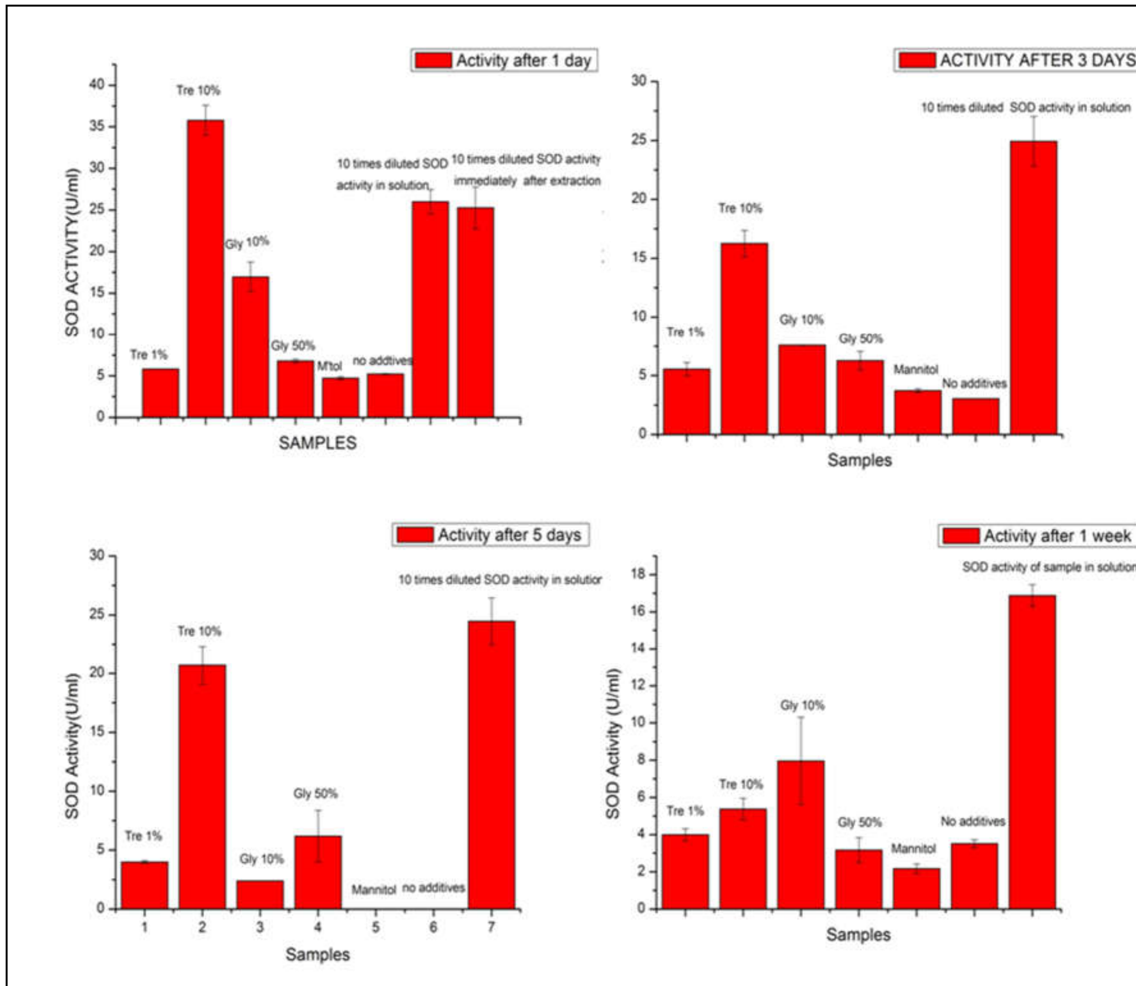

Figure 6. Accelerated shelf-life study. Data after first week of incubation at 60°C (equivalent to 10 weeks at RT).

at this temperature is equivalent to 5 weeks at room temperature), lower the freezing temperature to –20°C and test varying concentrations of the stabilizers.

Results are shown in Fig.6. Although, the best lyoprotectant – Trehalose 10%, performed better than the earlier mentioned concentration of 1%; even they showed only 10% retention of initial activity after 1 week, followed by Glycerol 10%; while others showed no activity and resulted in protein denaturation. Interestingly, one of our controls for the experiment, SOD extract in solution, showed good retention of enzyme activity for 5 days. Thereafter there was a sharp decrease in activity and only 7–8% of day 5 activity remained at the end of 1 week.

The possible explanation for the loss in activity of our freeze dried protein could be due to the fact that we had to return to –80°C freezing during this experiment, because at high

concentration of stabilizing agents the solutions hadn't frozen even after 3 days at –20°C and hence we reverted to the earlier protocol of –80°C for 3 hours. The extended freezing time could explain the loss of activity –~10% left after lyophilization while the control, i.e. SOD in solution, possibly lost activity as it was subjected to variations in experimental conditions due to its repeated usage during the various time points of the experiment.

Having observed good retention of enzyme activity in solution, we decided to compare 4 different samples: freeze dried protein stabilized using 10% Trehalose (frozen at –80°C for 3 hrs), SOD extract stabilized in solution with 10% trehalose, SOD in solution without any stabilizer and finally a control-extracted SOD stored at 4°C. We found that even without the stabilizer (i.e. trehalose 10%), there was some retention of activity while sample with 10%

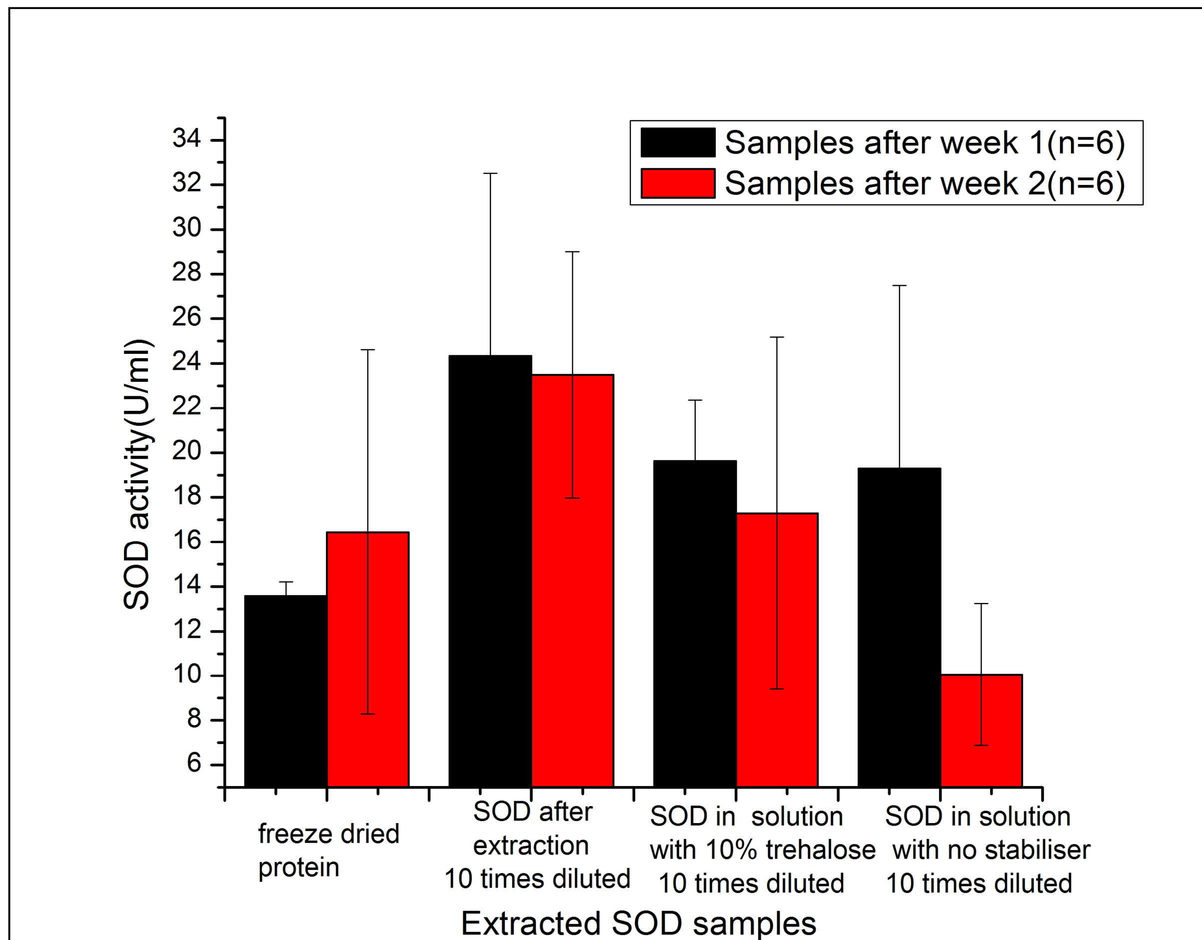

Figure 7. Accelerated shelf-life study. Data after first and second weeks of incubation at 50°C (equivalent to 10 weeks at RT).

trehalose showed very limited loss of activity (~75%(!) of the initial activity after extraction) after 2 weeks incubation at 50°C (which equals to 10 weeks at RT!)(Fig.7.). So, the major result obtained so far – the antioxidative enzyme concentrate extracted from porcine blood could be preserved without significant loss in antioxidant activity, even after 10 weeks at room temperature, when stabilized in solution using 10% Trehalose. Also, bypassing lyophilization/freeze-drying of the protein extraction, will have a tremendous impact on cost

reduction, as it does not require the additional capital associated with the setup and maintenance of commercial freeze driers; and the possible requirement of hiring a skilled technician to operate and maintain the equipment.

Our next step was to compare our enzyme concentrate to commercially available antioxidants, such as Pet Ox (BHA + BHT) and Nature Ox (mixed tocopherols), which we have received from ACREC. The another goal for second year was the shelf life study of the SOD concentrate in solution, since the preliminary data shows that it more stable in solution than freeze-dried. And the last part of the research for the second year was the estimation of costs, associated with extraction of SOD from animal blood in order to calculate the feasibility of commercializing the concept.

The objectives of this research are:

- To test antioxidant activity of our enzyme concentrate and compare its performance to that of currently used antioxidants;
- To study enzyme concentrate performance as food preservative after accelerated shelf life study (50°C, 2 weeks) and compare it to currently used antioxidants;
- To estimate costs, associated with extraction of SOD from animal blood.

We have focused on studying enzyme concentrate activity after accelerated shelf life study. In order to complete this goal, the SOD samples obtained from porcine blood, stabilized using the

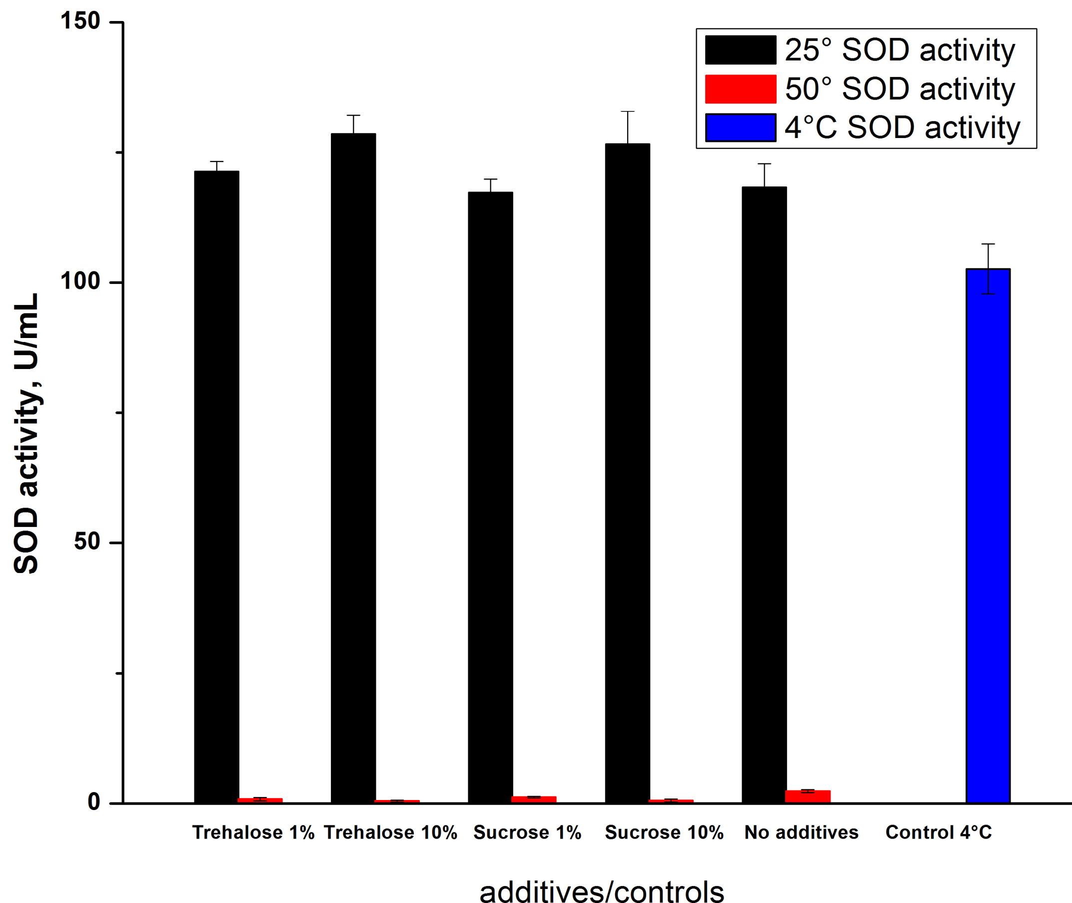

Fig.8. Results of SOD activity study for samples incubated for 20weeks at 50°C

sucrose (1 and 10% w/w), trehalose (1 and 10% w/w) and controls, were incubated at room temperature(22°C) and at 50°C (6 weeks at this temperature is equivalent to 1 year at room temperature). In our September report we showed data after 2 weeks of incubation at 50°C, and we continued those experiments further – after 8 weeks the samples stored at 25°C maintain their initial activity, and samples incubated at 50°C retained some activity (>60U/mL from initial 120U/mL) – which means that SOD concentrate is suitable for long term storage. The results of SOD assay after 20 weeks of incubation are shown on the Fig.8. As can be seen after 20 weeks at 50°C the enzyme lost its activity, while the concentrate stored at room temperature stays as active as it was initially (~120U/mL) – same as a control stored in the fridge. It should be noticed that all samples with different additives and without them showed similar activity, which means that no additive needed for prolonged storage at least up-to one year.

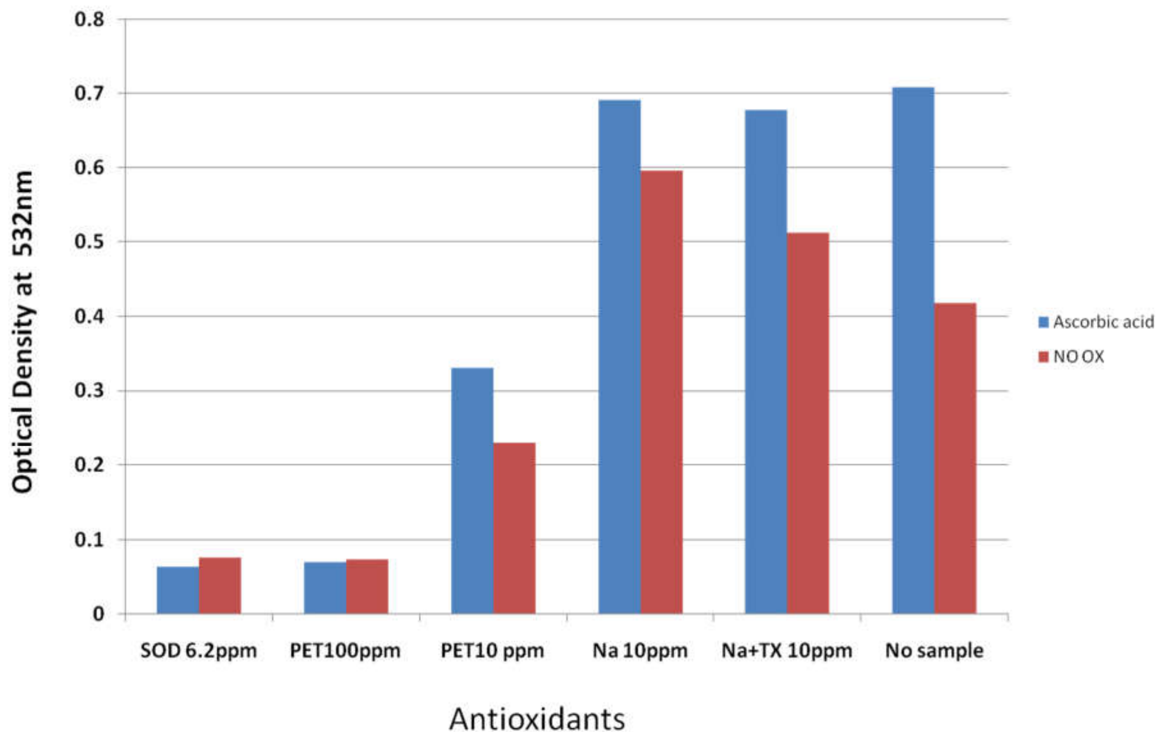

Figure 9. Results of TBA assay demonstrate that SOD concentrate works more effectively than 15-times-more concentrated PetOX and than NaturOx

Our next goal was to compare enzyme concentrate antioxidative properties. Common method to assess antioxidative properties of pet food additives is Oxidative Stability Index assay (OSI). The standard OSI assay isn't suitable for this system because it consists of oxidation of lipids by air flow at high temperatures (110-140°C) and the enzymes are not stable at high temperatures. The alternative assay Thiobarbituric Acid Reactive Substance assay (TBARS) is widely utilized. In this process, the sample is oxidized by either hydrogen peroxide or ascorbic acid/$FeCl_2$ and produced malondialdehyde (MDA), which later reacts with thiobarbituric acid forming colored adduct. The main result of the comparison was that the antioxidant concentrate we extracted

from animal blood showed **significantly higher activity** in much smaller concentrations than PetOX and Naturox. Fig. 9 shows the results of a TBARS assay: 6.2 ppm SOD concentrate worked more effectively than 100 ppm PetOX (15 times more concentrated) and much more effectively than NaturOx. This amount, 6.2 ppm, of SOD can be extracted from 31mL of animal blood.

Our research further showed that enzyme concentrate extracted from animal blood extinguished anti-peroxide activity and anti-superoxide activity. We compared its antioxidative properties to highly-purified commercial bovine erythrocyte SOD using a TBA assay; our SOD concentrate showed much higher activity, probably because of the presence of catalase. According our data, the peroxidase (catalase) activity of the enzyme concentrate is ~125U/mL. Because of the synergistic effect of the two enzymes, this product can more effectively protect against oxidation. By the end of the second year of the project, we estimated the costs associated with extraction of SOD from animal blood to calculate the feasibility of commercialization.

Currently used 1000ppm of PetOx, main component BHA+BHT: price is $20-450 per kg (source alibaba.com, PetOx from Kemin Inc. – more expensive!). The price per ton is therefore **$20-100 per ton** of animal feed.

Estimate for SOD: The same level of antioxidant activity can be achieved with:

- 10 times lower concentration than PetOx;
- Only 0.5L of blood per ton of feed;
- So, **the estimated price** per ton of animal food is **$1.5/ton ***

	Working Concentration	Estimated price per ton of meals
BHA+BHT	1000 ppm	$20-100
Crude SOD	100 ppm	**$1.5***

(*) based on bulk chemicals pricing and w/o production cost

Conclusions:

During first two years of this project we developed a working protocol for SOD extraction from different types of animal blood with high extraction yield and activity of final product. We also studied the effect of stabilizers and cryoprotectors and the shelf life of samples in solution and after freeze-drying procedure. We found that while freeze-dried protein lacking of activity after 1 week storage at high temperature, but at the same time the activity of enzyme stored in solution remained high even after 8 weeks stored at 50°C and after 20 weeks stored at room temperature. TBA assay performed on SOD samples and compared to currently used antioxidants – PetOx and NaturOx demonstrated that 6.2 ppm SOD concentrate worked more effectively than 100 ppm PetOX (15 times more concentrated!) and much more effectively than NaturOx.

Impacts and Significance:

Recently, use of enzymes in the food industry has rapidly increased. However, considering the total number of functional enzymes so far described by biochemists, only a handful are presently in commercial use. Animal co-products, such as blood and intestines, are characterized by high protein content and could be a valuable source of enzymes. The proposed project, a continuation of our current research, will compare the antioxidant capacity of extracted SOD concentrate to antioxidants currently used in the pet-food and livestock-feeds industries and evaluate the feasibility of commercialization.

Since SOD concentrate is at least 15 times more effective than the best of commercial antioxidants, PetOx, it can be used in smaller concentrations. According to the MSDS, PetOx is usually added to pet food at 2lb/ton (1000ppm). The amount of SOD for a concentration of 100ppm can be extracted from 0.5L of blood. Based on bulk pricing, the estimated cost of the chemicals to be used for the extraction is ~$3/1L of blood.

Interest in substituting synthetic-food preservatives and synthetic antioxidants for substances that can be marketed as natural is increasing. Pet-food and livestock-feed preservatives based on antioxidant enzymes extracted from animal blood (mainly SOD) should have a huge impact on the industry due to the following proven properties of SOD:

- Prevents lipid oxidation in food, thus preserving nutritional value and palatability
- Has anti-aging properties that provide an additional source of a valuable enzyme, thus reducing the body's need to form amino acids ;
- SOD administrated orally has been shown to prevent the tumor progression that inflammation promotes (Okada et al., 2006).

During the first two years of the project, we demonstrated the high level of SOD activity in enzyme concentrate. When fully developed, the process of SOD extraction will be simple and cost efficient. The product can be used as an antioxidant supplement to animal feed and, as such, will have a large market. Although we focus on animal-feed-supplement applications due to less rigorous regulatory requirements, SOD has a broader range of applications (e.g., as preservative in foods or as a drug) that can provide additional markets for the product to be developed in this project.

Publications:
None

Outside funding:
We are planning commercialization of this technology, and therefore had to take care of IP protection prior to seeking outside funding sources. Patent application has recently been filed by Clemson. Funding will be sought through licensing of this technology, with the help from Clemson Technology Transfer Office. If there is no immediate interest from the rendering industry to license

this technology, we plan to start up a company and seek funding from federal agencies (USDA being the primary target agency) using SBIR mechanism. Other potential funding source is SC Launch program, which provides $50,000 seed grants to start up businesses in SC with high funding rate and matches SBIR grants 1:1.

Future Work:

Future work should be focused on evaluation of SOD concentrate properties in real-life application and development of a small-scale production protocol.

Acknowledgments:

PIs are extremely grateful to FPRF for funding and important guidance, Dr. Annel Greene – for her help and constant support, Laine Chambers, and our students Sriram Sankar, Chengyi Tu, Victor Dipiero.

References:

1. Food chemistry / H.-D. Belitz, W. Grosch. Berlin ; New York : Springer, c1999.
2. Proteins in food processing / edited by R.Y. Yada. Cambridge, England : Woodhead Publishing ; Boca Raton, FL : CRC Press, 2004
3. Enzymes in food processing / edited by G.A. Tucker and L.F.J. Woods, London ; New York : Blackie Academic & Professional, c1995.
4. Enzymes in food technology / edited by Robert J. Whitehurst and Barry A. Law., Sheffield : Sheffield Academic Press ; Boca Raton, FL : CRC Press, 2002.
5. Minimal Processing Technologies in the Food Industry Edited by: Ohlsson, T.; Bengtsson, N., Woodhead Publishing, 2002
6. Kumagai, Y., Shinyashiki, M., Sun, G. F., Shimojo, N., and Sagai, M. (1994), *Experientia* **50**, 673–676
7. Y. Mercier, P. Gatellier, M. Renerre, Lipid and protein oxidation in vitro, and antioxidant potential in meat from Charolais cows finished on pasture or mixed diet, Meat Science, Volume 66, Issue 2, February 2004, Pages 467-473.
8. US Patent 3920521 Michelson A.M. et al., Superoxide dismutases and their application as oxidation inhibitors, 1974
9. Heinrich, Peter; Georg Löffler; Petro E. Petrides (2006). *Biochemie und Pathobiochemie (Springer-Lehrbuch) (German Edition)*. Berlin: Springer. pp. 123. ISBN 3-540-32680-4.