FINAL REPORT
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Livestock feed preservatives based on antioxidant enzymes extracted from animal blood

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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 establish a simple method to produce protein 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: Autooxidation of unsaturated fats is one of the primary mechanisms of quality deterioration in animal feed. These alterations in quality are manifested through adverse changes in flavor, color, texture, and nutritive value and there is some concern that toxic compounds are produced during the deterioration process. The pet food industry constantly looks for natural solutions to increase the palatability of livestock feeds and **pet food,** and also studies mechanisms to extend the shelf life of products while maintaining product freshness and quality. Most currently used antioxidants are synthetic chemicals, and there is global concern among regulatory bodies and customers regarding the safety of these compounds (e.g., ethoxyquin). Naturally-derived antioxidants are available (e.g., tocopherols), but are expensive, and are often not as effective as their synthetic counterparts. Thus, there is a clear need for development of novel, inexpensive, and efficient natural antioxidants. Erythrocytes are readily available as a major component of animal blood. Being natural oxygen carriers, they are equipped with highly efficient antioxidant machinery, which utilizes a number of antioxidant enzymes and compounds. Here, we propose the continuing development of a cost effective, natural antioxidant derived from animal blood.

During Year 1 of the project, we established a simple protocol to produce an antioxidant enzyme concentrate from animal blood. Throughout the project's second year, we compared the efficacy of our enzyme cocktail to commercially available antioxidants, such as PetOx® (BHA + BHT) and NaturOx® (mixed tocopherols), and completed accelerated shelf-life studies. During the 3rd year we have made major progress by establishing a **new extraction protocol** which eliminates the production stages involving organic solvents, thus decreasing the cost of the final

product. By the end of Year 3, we estimated the costs associated with the newly developed procedure for antioxidant extraction from animal blood.

Materials and methods. Fresh porcine blood was generously donated by Wilson Processing, Inc. (Seneca, SC). Chloroform was purchased from VWR Inc. (West Chester, PA). HPLC Grade Acetone, anhydrous dipotasium hydrogen phosphate (K_2HPO_4), phosphate buffered saline tablets (PBS), sodium chloride were obtained from Fisher Scientific Inc. (Fair Lawn, NJ). Pierce BCA protein assay kit was bought from Thermo Scientific Inc. (Rockford, IL). WST-1 was from Dojindo Molecular Technologies Inc. (USA). Xanthin/Xanthine and Catalase (from bovine liver) was bought from Sigma-Aldrich, LLC. (St. Louis, MO). Superoxide dismutase (bovine erythrocytes) was from Calbiochem Inc. (Darmstadt, Germany). Xanthine Oxidase (from cow milk) was purchased from Roche Diagnostics Inc. (Mannheim, Germany). Hydrogen peroxide (H_2O_2 , 3%) was purchased from Rite Aid (Camp Hill, PA).

Extraction of crude SOD (C-SOD) by the standard method. The standard method to extract SOD from blood was adopted with minor modifications. In brief, fresh porcine blood was centrifuged at 5000xg for 15 min to obtain packed red blood cells (RBCs), followed by washing using 0.9% sodium chloride twice. The clean and packed cells were lysed by equal volume of 1% tween-20 in DI water. Hemoglobin was then removed by Tsuchihashi treatment (ethanol/chloroform 62.5/37.5 v/v). Then, salting out was carried out with K_2HPO_4 (1/3 w/w) to remove extraneous proteins. Lastly, acetone (75% v/v) was applied to precipitate and obtain crude SOD. The resulting product was washed briefly by 1X PBS and re-dissolved in the same buffer with minimum volume.

Extraction of crude protein mixture (CP) by a simplified method. Briefly, fresh porcine blood was centrifuged at 5000g for 15min to obtain a pellet of packed RBCs. Subsequent cell lysis was conducted with 3-4 times cell volume of DI water, followed by vigorous shaking for 30 min to ensure complete lysis. Hemoglobin-precipitating agent was then added, followed by incubation and occasional shaking for about 1hour or longer. The hemoglobin was precipitated and removed by centrifugation at 5000g for 15min. Resulting transparent solution was stored refrigerated (4°C).

Total protein content analysis. The total protein contents of C-SOD and CP were determined by bicinchoninic acid (BCA) assay using the assay kit purchased from Pierce Biotechnology Inc. Its basic principal is as follow. In an alkaline solution Cu^{2+} is reduced to Cu^{1+} by proteins, and bicinchoninic acid specifically complexes with cuprous cation (Cu^{1+}) and the resulting compound strongly absorbs at 562 nm. In brief, 50 parts of solution "A" is mixed with 1 part of solution "B" to obtain working solution. Then 0.1 ml of sample was mixed with 2.0 ml of working solution, followed by incubation at 37 ℃ for 30 min. The absorbance at 562 nm of the resulting solution was measured using a microplate reader. Total protein contents were calculated by comparing with the standard curve.

SOD activity assay. SOD activity was determined by the xanthine-xanthine oxidase method. This method is based on a water soluble tetrazolium salt, WST-1 which is converted into formazan dye upon interaction with superoxide anion generated by the xanthine/xanthine oxidase system. Therefore, SOD activity is reversely proportional to the production of the yellow formazan dye. A 20 µl aliquot of each sample (n=3) was mixed with reaction reagents containing xanthine, xanthine oxidase and WST-1 in 96-well plate (Costar). The reaction was carried out at 37 ℃ for 1hour. The absorbance at 450 nm was read with a microplate reader. SOD activity was calculated based on the standard curve (0.1 U/ml - 100 U/ml).

Catalase activity. Catalase activity was determined using the method described by Pardha. In brief, each sample (0.1 ml) was reacted at room temperature with 2.9 ml of 30 mM hydrogen peroxide in HEPES buffer (pH=7.0). The loss of H_2O_2 was continuously monitored by measuring the absorbance at 240 nm. A unit of catalase would decompose 1 µmole of H_2O_2 per minute at room temperature at pH 7.0.

Results

The results have been summarized in **Table 1**. The two extraction methods started with same volume (100 ml) of blood so they would be directly comparable. The standard method yielded 20 ml of crude SOD solution (C-SOD) with protein concentration of 2.2 mg/ml. In comparison, the simplified method obtained 91 ml of final solution (CP) with protein concentration of only 0.6 mg/ml. It can be calculated from these numbers that the total protein yields for C-SOD and CP are 44.0 mg and 54.6 mg respectively.

In terms of SOD activity, C-SOD was much more concentrated than CP, with SOD activity of as high as 1100 units/ml compared to 224 units/ml of CP. Further, considering that the standard method was specifically designed to extract SOD, it was not surprising C-SOD also showed a higher SOD specific activity (500 units/mg) over CP (373 units/mg). However, it should be noted that the total SOD activity yields for the two methods are close (22000 units for C-SOD and 20384 units for CP from 100 ml of blood).

Table 1 Characterization of C-SOD and CP

	Initial blood volume (ml)	Ending volume (ml)	Protein concentration (mg/ml)	SOD activity (U/ml)	SOD specific activity (U/mg)	Catalase activity (U/ml)
C-SOD	100	20	2.2	1100	500	300
CP	100	91	0.6	224	373	<10

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 by-products, such as blood and intestines, are characterized by high protein content and could be a valuable source of enzymes.

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 should have a huge impact on the industry.

During the previous years of the project, we demonstrated the high level of antioxidative activity in enzyme concentrate. 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, vegetable oils or as a drug) that can provide additional markets for the product to be developed in this project.

Publications: None

Outside funding: We're planning to submit an application for NSF I-CORPS this Fall with Dr. David Meisinger serving as an industrial mentor. Our team is being considered for the cohort in Jan-February, 2014. We're also planning to submit USDA SBIR proposal this September.

Future Work:

Identify the proper type of pet food to which the erythrocytes extracts, C-SOD and CP, can be applied. Test the samples in pet food in a real industrial manufacturing, packaging and storage conditions. Though CP and C-SOD were demonstrated to be effective in the two models in the experiments, the real industrial conditions can be more complicated. Moisture, UV irradiation and presence of pro-oxidants are all reasons for fast oxidation of fats while it is unknown whether the erythrocytes extracts can successfully work against all of them. They need to be tested on the real pet food in the manufacturing facility to validate their efficacy.

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