Extraction of Superoxide Dismutase, Catalase and Carbonic Anhydrase from stroma-free red blood cell hemolysate for the preparation of the nanobiotechnological complex of PolyHemoglobin-Superoxide Dismutase-Catalase-Carbonic Anhydrase

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Abstract

We report a novel method to simultaneously extract superoxide dismutase (SOD), catalase (CAT) and carbonic anhydrase (CA) from the same sample of red blood cells (RBC). This avoids the need to use expensive commercial enzymes thus allowing this to be cost effective for large-scale production of a nanobiotechnological polyHb-SOD-CAT-CA with enhancement of all 3 red blood cell functions. The best concentration of phosphate buffer for ethanol-chloroform treatment results in good recovery of CAT, SOD and CA after extraction.Different concentrations of the enzymes can be used to enhance the activity of polyHb-SOD-CAT-CA to 2, 4 or 6 times that of RBC.

Introduction

Polyhemoglobin (polyHb) is the first generation blood substitute prepared by the basic method of cross-linking the hemoglobin molecules with glutaraldehyde[Chang 1971]. This basic principle was later developed independently by 2 U.S. groups and tested in clinical trials [Jahr et al, 2008; Moore et al 2009, Kim & Greenburg 2014.]. Bovine PolyHb has been approved for use in patients in South Africa and Russia [Kim & Greenburg, 2014]. U.S. Clinical trials on ambulance hemorrhagic shock patients shows that human PolyHb can be given on the spot without blood typing and can delay the need for blood transfusion for 12 hours while the saline control group requires blood transfusion within 1 hour [Moore et al, 2009]. There were 3% nonfatal cardiac side effects compared with 0.6% in the control group. The investigators propose that it has potential in fatalhemorrhagic shock when donor blood is not available [Moore et al 2009]

Normal red blood cell (RBC) has three main functions: delivery of oxygen to tissues and cells; remove oxygen radicals; and transport carbon dioxide from tissues to the lungs [Chang 2007]. Ischemia-reperfusion injuries may happen in severe hemorrhagic shock due to the production of oxygen radicals during reperfusion with transfusion[D'Agnillo & Chang, 1998, Alayash, 2004]. The original blood substitutes were prepared from red blood cell stroma-free hemolysate (SFHb) that contains all the red blood cell enzymes of SOD,CAT and CA [Chang 1964, 1971].In order to enhance the antioxidant enzyme activities, we have added higher concentrations of the enzymes,

SOD and CAT to prepare PolyHb-SOD-CAT[D'Agnillo & Chang, 1998]. This has prevented ischemia-reperfusion in a hemorrhagic shock stroke rat model (Powanda and Chang 2002]. In hemorrhagic shock, the mortality and the severity of myocardial ischemia are related to the elevated intracellular pCO_2[Sim et al, 2001]. The increase of intracellular carbon dioxide level may lead to acidosis, malfunction of central nervous system and even death[Geers & Gros 2000]. The red blood cell enzyme carbonic anhydrase (CA) is the most important component for the transport of CO_2 [Geers & Gros, 2000; Arthurs & Sudhakar 2005]. This led this laboratory to prepared a even more up to date nanobiotechnological complex of polySFHb-SOD-CAT-CA with higher concentrations of all 3 enzymes than those normally present in red blood cells [Bian,Rong & Chang 2012; Bian & Chang 2015]. This polySFHb-SOD-CAT-CA complex with enhanced RBC enzyme activities has been tested in a 90 min hemorrhagic shock rat model with 2/3 blood volume loss. The result shows that it is more effective than whole blood in lowering the elevated intracellular pCO_2, preventing cardiac side effects and preventing ischemia-reperfusion injuries in the liver, intestine and the heart [Bian & Chang 2015].

Stroma-free hemolysate (SFHb) is the content of RBC with the membrane removed. This contains hemoglobin and RBC enzymes including CAT, SOD and CA. SFHb containing these enzymes has been used to prepare the earlier blood substitutes [Chang 1964, 1971]. However, even RBC with its normal CAT, SOD and CA enzyme activities is not sufficient to prevent irreversible shock in serious sustained hemorrhagic shock[Kim & Greenburg 2014; Bian & Chang 2015]. We therefore successfully prepared polySFHb-SOD-CAT-CA complex by crosslinking SFHB with additional CAT, SOD and CA [Bian, Rong & Chang, 2012, Bian & Chang, 2015]. However, commercial CAT, SOD and CA are too expensive for scale up production towards potential clinical use. Gu and Chang [2009] have extracted SOD and CAT from SFHb. Ethanol-chloroform was used to denature the hemoglobin then acetone precipitation was used to purify and precipitate the SOD. Carbonic anhydrase can also be extracted by ethanol-chloroform treatment and acetone precipitation method[da Costa Ores et al 2012]. However, this method results in the inactivation of CAT. Thus CAT had to be precipitated separately by adding ammonium sulfate[Gu & Chang 2009]. The effect of buffer on the stability of proteins has been studied by Ugwu and Apte[2004], They show that phosphate buffer pH 7.4 promotes the conformational stability of enzymes. Therefore, we use potassium phosphate buffer (PPB) to protect CAT in ethanol-chloroform treatment in the present study. Extracting individual enzymes from different batches of SFHb is neither efficient nor cost effective. In the present study we have designed an optimal method for extracting all three enzymes at the same time from the same sample of SFHb.

In this study, we successfully establish the method to extract CAT, SOD and CA from blood and crosslink them with SFHb to prepare PolySFHb-SOD-CAT-CA. The use of different concentrations of Potassium Phosphate Buffer (PBS) for the extraction was analyzed in details. As a result, we are able to establish the optimal concentration of potassium phosphate buffer for the extraction of CAT together with SOD and CA. When adding 3M PPB to SFHb, CAT activity can be retained in the Hb-free supernatant after ethanol-chloroform treatment; SOD and CA can also be obtained simultaneously. Finally, the extracted enzymes would be purified and concentrated, followed by crosslinking them with SFHb to generate polySFHb-SOD-CAT-CA. Our results show that our modified ethanol-chloroform treatment can maintain CAT, SOD and CA activities with high recovery yields. The method we established is expected to cut down the costs of scale up production compared with the use of commercial enzymes.

Materials and Methods

Materials

Stroma-free hemoglobin (SFHb) was prepared from bovine blood purchased from McGill University McDonald Campus Cattle Complex (Sainte-Anne-de-Bellevue, Canada). Both Amicon

Ultra-15 Centrifugal Filter Unit with Ultracell-10 membrane and Biomaxpolyethersulfone ultrafiltration disc with 300 kDa cut off were purchased from Millipore. All other experimental and analytical reagents were purchased from Sigma.

Preparation of SFHb

The method was based on that of Chang [2007]. Briefly, fresh heparinized bovine blood was centrifuged at 4,500g for 30 minutes at 4℃. The plasma supernatant containing white blood cells and platelets were aspirated carefully on ice leaving the red blood cells (RBCs). Wash the RBCs with 0.9M sodium chloride three times. RBCs were then lysed by adding 2 times volume of hypotonic sodium phosphate buffer (12.5mM, pH 7.4) to 1 volume of RBCs. The solution was mixed by repeated inversion and swirling, then left standing for 30 minutes at 4℃. 0.5 volume of cold toluene was added to the mixture. After shaking vigorously, the supernatant was allowed to stand for 3 hours at 4℃. The upper layer of the mixture which contained toluene, stromal lipid and cellular debris was removed by aspiration. The toluene step was repeated one more time, and the remaining solution was centrifuged at 16,000g for 2 hours at 4°C. The supernatant was then filtrated by paper filter. Toluene was removed by vacuum. The stroma-free hemoglobin solution was mixed by stirring, and distributed into 15ml centrifugal tubes and stored at -80℃.

Quantitative determination of Hb concentration

The Hb concentration is colorimetrically determined by reaction of the samples with Drabkin's reagent (Sigma-Aldrich). After 12μl sample was added to 3ml Drabkin's reagent in cuvette, the solution was allowed to stand for 15 minutes at room temperature, avoiding the light. The concentration of cyanmethehemoglobin in solution was measured by spectrophotometry at 540 nm.

Determine of CAT activity

The established method was described in the paper of Zhu and Chang [21]. The SFHb and polySFHb samples were diluted into 1:200 with potassium phosphate buffer (50mM, pH 7.0) to total volume of 5ml;while the SFHbmixed with extracted enzymes and polySFHb-SOD-CAT-CA samples were diluted into 1:1000. The H_2O_2 solution was prepared by adding 40.6μl30% H_2O_2solution to 9ml potassium phosphate buffer (50mM, pH 7.0). UV 240nm spectrophotometer was used to measure the rate of elimination of H_2O_2within 15 seconds. 1ml diluted sample and 0.5ml buffer were mixed for blank. Then 1ml dilutedsample mixed with 0.5 ml H_2O_2solution was applied to determine the CAT activity in the sample.

Determine of SOD activity

The SOD activity assay measures the decrease of cytochrome c by superoxide at UV spectrophotometer 550nm within 50 seconds [11]. The SFHb and polySFHb samples were diluted 20 times with potassium phosphate buffer (0.1M, pH 7.4); while the SFHb mixedwith extracted enzymes and polySFHb-SOD-CAT-CA samples were diluted into 100 times. The reaction buffer prepared consists of potassium phosphate (50mM, pH 7.8), EDTA (10^{-4}M), xanthine (5×10^{-5}M), cytochrome c (10^{-5}M), and catalase (500U/ml). Xanthine oxidase was diluted with 0.154M NaCl to 6U/ml. The mixture of 1.45ml buffer and 25μldiluted sample or buffer was applied as blank, and 25μl xanthine oxidase (6U/ml) was then added to initiate the reaction.

Determine of CA activity

The procedure for CA measurement is based on the established method by Bian 2012 [17]. Tris buffer (20mM, pH 8.3) and diluted samples as above were prepared. The substrate of the reaction was prepared by bubbling CO_2 through distilled water. 10μl diluted sample or the same

amount of buffer as control was added into 3ml Tris buffer with the monitor of pH changes. 2ml of saturated CO_2solution was added to start the test. The time needed for the pH of mixture drop from 8.3 to 6.3 was recorded.

Molecular weight distribution

Sephacryl-300 HR columnat flow rate of 130 ml/hour was used for the analysis of molecular weight distribution. The column was equilibrated with 0.9%NaCl buffer. After 200μl sample with Hb concentration of 9g/dL was loaded into the column, 0.9%NaCl buffer was used to elute the sample. The molecular weight distribution was recorded by a 280nm UV detector at velocity of 5mm/min.

Results

Effects of Potassium Phosphate Buffer concentrations on Enzyme Extraction

For this we followed the procedure of enzyme extraction from hemolysate described in the next section except that we used different concentrations of potassium phosphate buffer PPB (pH 7.4):

1 SFHb 3ml + 4M PPB 0.6ml + ethanol-chloroform 1.2ml
2 SFHb 3ml + 3M PPB 0.6ml + ethanol-chloroform 1.2ml
3 SFHb 3ml + 2M PPB 0.6ml + ethanol-chloroform 1.2ml
4 SFHb 3ml + 1M PPB 0.6ml + ethanol-chloroform 1.2ml
5 SFHb 3ml + 0.1M PPB 0.6ml + ethanol-chloroform 1.2ml
6 SFHb 3ml + 0.05M PPB 0.6ml + ethanol-chloroform 1.2ml
7 SFHb 3ml + water 0.6ml + ethanol-chloroform 1.2ml
8 SFHb 3ml + ethanol-chloroform 1ml

TABLE I . Recovery yield of apparent activities of CAT, SOD and CA after extraction using different concentrations of phosphate ions (See discussion regarding the possible reasons for the higher recovery of CAT activity)

	4M PPB	3M PPB	2M PPB	1M PPB	0.1M PPB	0.05M PPB	Water	Nothing
Final PO_4^{2-} Conc. (mM)	507.8	382.8	257.8	132.8	20.3	14.05	7.8	9.375
CAT recovery yield (%)	194±41	209±65	204±56	118±23	6±3	3±2	0	11±7
SOD recovery (%)	86±7	88±5	70±10	70±3	77±9	80±8	98±9	80±12
CA recovery (%)	80±10	102±5	74±7	76±5	84±2	84±7	95±4	93±10

Table I summarized the results obtained. The enzyme activity in the SFHb was considered as 100%. For CAT, there was little or nor enzyme recovered at the lower phosphate buffer concentrations. Increase in the concentration of phosphate ions resulted in increasing recovery of CAT with the highest yield at 3M PPB. The reasons for the more than 100% CAT recovered apparent activity will be explained under discussion. The recovery yields of SOD and CA were both around 70-100%, compared with the activities in SFHb. The concentration of phosphate buffer had no effect on the recovery yields of SOD and CA.

Based on these results, we used 3M for the final procedure of enzyme extraction as described in the next section.

Final method established for the Extraction and Concentration of Enzymes from Stroma-free Hemolysate

Add 20ml SFHb to a beaker on ice followed by 4ml 3M Potassium Phosphate Buffer (PPB pH

7.4) to a final concentration of 9g/dL SFHb. Then stirred using magnetic stirrer with medium speed for 5mins on ice. Add drop-wise 8ml cold ethanol-chloroform solution with a speed of 1 drop/sec. Stir for 30 minutes with medium speed on ice. Centrifuge the solution for10mins at 5000rpm at 4°C. Collect the supernatant layer (usually around 20ml) which contained recovered SOD, CA, and CAT with little or no Hb. Transfer the supernatant to a new centrifugal tube, and centrifuge at 5000rpm for 10mins at 4°C to remove any remaining precipitated Hb in the supernatant. Transfer 12 ml of the supernatant to Amicon Ultra-15 Centrifugal 10 kDa Filter Unit and centrifuge 10 minutes at 6000rpm at 4°C. Add remaining 8-10ml of supernatant to the same filter unit containing the enzyme solution and centrifuge at 6000rpm for 15mins at 4°C.Wash out the remaining ethanol in the extracted enzyme solution by adding 5ml 0.2M $Na{\cdot}PO_4$ Buffer (pH 7.4) to centrifugal unit, mix the solution, and then centrifuge at 6000rpm at 4°C for 15 minutes. Repeat the washing steps 2 more times. Finally, to minimize the final volume of concentrated enzymes, wash the enzymes solution with 2ml $Na{\cdot}PO_4$ Buffer (pH 7.4) and centrifuge at 6000rpm at 4°C for 15mins. Final volume of concentrated enzymes should be approximately 300µl.

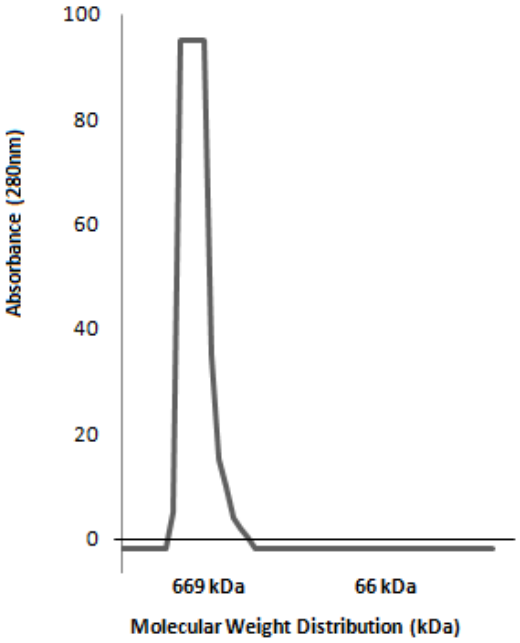
Final method established for the preparation of PolySFHb-SOD-CAT-CA using Extracted Enzymes

Add 4ml of 9g/dl SFHb to different amounts of the concentrated enzymes prepared as described above. Then add 102µl 4M NaCl to the solution. Shake at 160rpm at 4°C for 5mins.Frozen glutaraldehyde (0.5M) was thawed on ice water mixture by ultrasonic machine for 30-40mins. Mix glutaraldehyde evenly before use. Without stopping the shaker, add drop-wise 162.3µl glutaraldehyde in 4 equal aliquots at intervals of 15mins. Shake at 160rpm for 24hrs at 4°C. Add 641.4µl 2M lysine (200:1 lysine to protein molar ratio) over 2 equal aliquots over 10 minutes and shake at 160 rpm at 4°C for 1 hour to quench. Centrifuge at 8000rpm at 4°C for 1 hour to remove any precipitate. The preparation of polySFHb followed the same procedure as for the preparation of polySFHb-SOD-CAT-CA but without adding the extracted enzymes. The supernatant of cross-linked samples were around 4-5ml. The sample was purified using 300KDa ultrafiltration disc with positive air pressure. After purification, the Hb concentrations of the crosslinking samples were tested and adjusted to 9g/dL with 0.1M potassium phosphate buffer (pH 7.4).

Molecular Weight Distribution

The molecular weight distributions of polySFHb-SOD-CAT-CA, polySFHb and SFHb were analyzed with Sepacryl S-300 gel column chromatography. 200µl supernatant was load on the column. In the polymerized products polySFHb-SOD-CAT-CA or polySFHb, most of the molecules were in the >669KDa range (Fig. 1 A). For SFHb most of the molecules were in the 64KD hemoglobin range (Fig. 5 B).

(A)



(B)

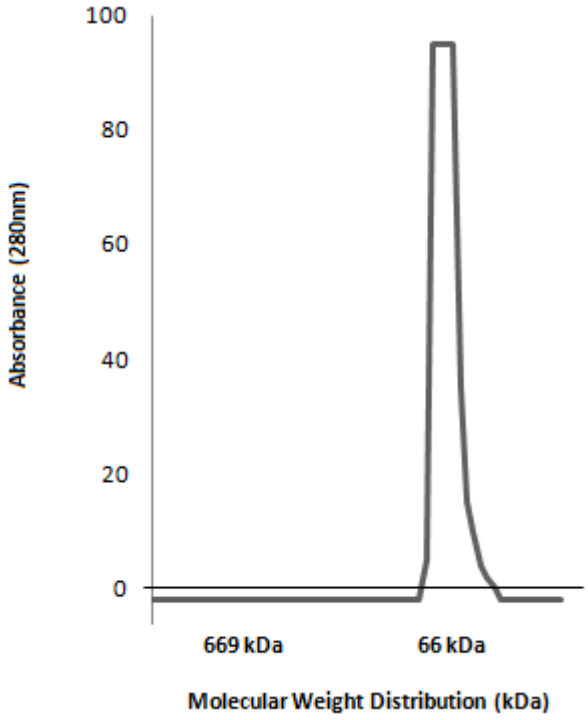


Fig 1. (A) The molecular weight distribution of polySFHb-SOD-CAT-CA or polySFHb. (B) The molecular weight distribution of SFHb.

Determine of Enzyme Activities and Recovery Yield

The CAT, SOD and CA activities were measured for the following samples before and after crosslinking. SFHb, SFHb with concentrated enzymes added at 2x, 4x and 6x the original SFHb enzyme concentrations. After purification of the polymerized molecules with 300KDa ultrafiltration disc, the enzymes activities of the complexes were measured (Figures 2-4)

Catalase activity in SFHb is 53234±2945U/gHb. After crosslinked into polySFHb the catalase activity was 20124±1053U/gHb). SFHb plus different amount of concentrated extracted enzymes

before crosslinking and after crosslinking also show good recovery of catalase activities (figure 2). For example, the catalase activity for SFHb with 2x extracted SOD, CAT and CA was 153046±2207U/gHb before crosslinking and 93034±3537U/gHb after crosslinking. 4x extracted SOD, CAT and CA was 160320±2205 U/gHb) before crosslinking and 135909±3918 U/gHb after crosslinking. 6x extracted SOD, CAT and CA was 326989±7385U/gHb) before crosslinking and 273919±7379U/gHb after crosslinking

Superoxide dismutase activity in SFHb is 1642±48U/ml. After crosslinked into polySFHb the catalase activity was 1114±33U/ml (Fig.3). In SFHb plus different amount of concentrated extracted enzymes before crosslinking and after crosslinking also showed good recovery of SOD activities (figure 4). For example, the SOD activity for SFHb with 2x extracted SOD, CAT and CA was 3567±100U/gHb) before crosslinking and 2846±68U/gHb after crosslinking. 4x extracted SOD, CAT and CA is 4526±65U/gHb) before crosslinking and 4149±69 U/gHb after crosslinking. 6x extracted SOD, CAT and CA was 9124±338U/ml before crosslinking and 6712±181U/ml after crosslinking.

Carbonic anhydrase activity in SFHb was 16725±411U/gHb. After crosslinked into polySFHb the catalase activity was 18638±711U/gHb (Fig.4). In SFHb plus different amount of concentrated extracted enzymes before crosslinking and after crosslinking also showed good recovery of SOD activities (figure 5). For example, the CA activity for SFHb with 2x extracted SOD, CAT and CA was 25722±758 U/gHb) before crosslinking and 24547±574 U/gHb after crosslinking. 4x extracted SOD, CAT and CA was 62776±680 U/gHb before crosslinking and 54707± 400U/gHb after crosslinking. 6x extracted SOD, CAT and CA was was 140243±2911U/gHb before crosslinking and 101869±4248U/gHb after crosslinking.

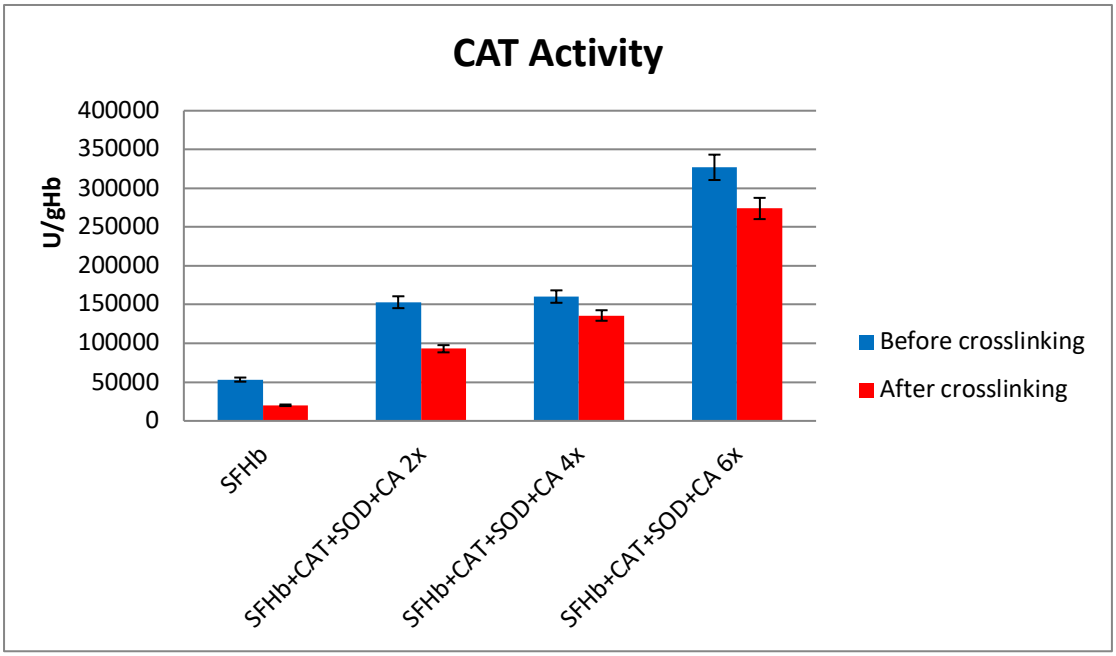


Figure 2: Comparison of CAT activities in different samples. Left to right: SFHb before and after crosslinking; SFHb+CAT+SOD+CA 2x (SFHb plus 2x extracted SOD, CAT and CA) before and after crosslinking; SFHb+CAT+SOD+CA 4x (SFHb plus 4x extracted SOD, CAT and CA) before and after crosslinking; SFHb+CAT+SOD+CA 6x (SFHb plus 6x extracted SOD, CAT and CA) before and after crosslinking.

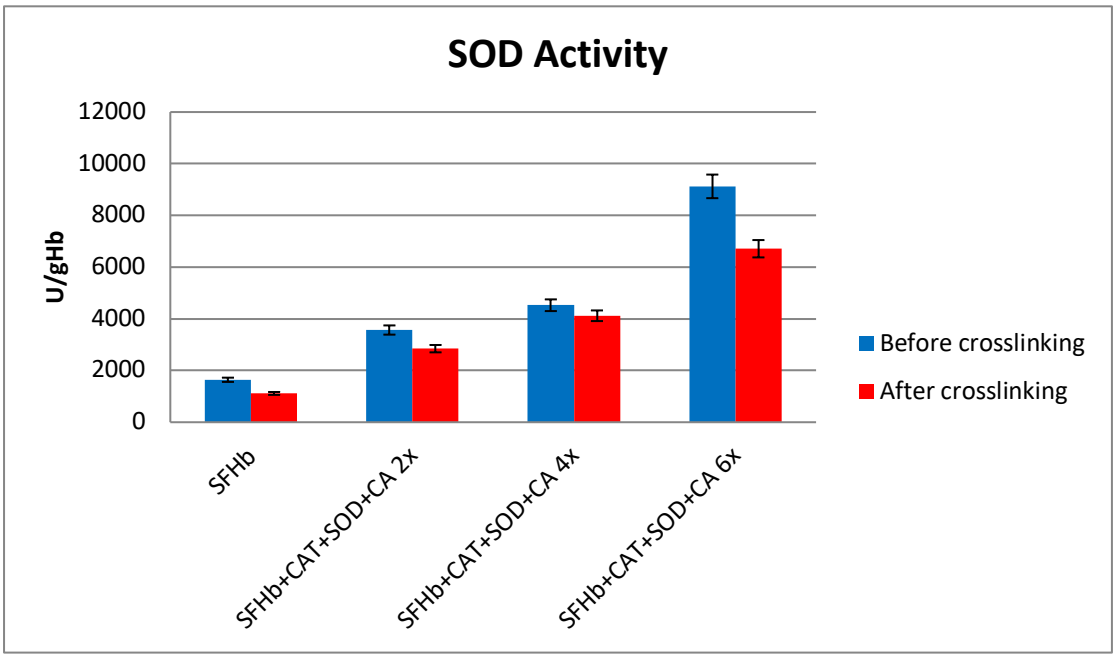


Figure 3: Comparison of SOD activities in different samples. Left to right: SFHb before and after crosslinking; SFHb+CAT+SOD+CA 2x (SFHb plus 2x extracted SOD, CAT and CA) before and after crosslinking; SFHb+CAT+SOD+CA 4x (SFHb plus 4x extracted SOD, CAT and CA) before and after crosslinking; SFHb+CAT+SOD+CA 6x (SFHb plus 6x extracted SOD, CAT and CA) before and after crosslinking.

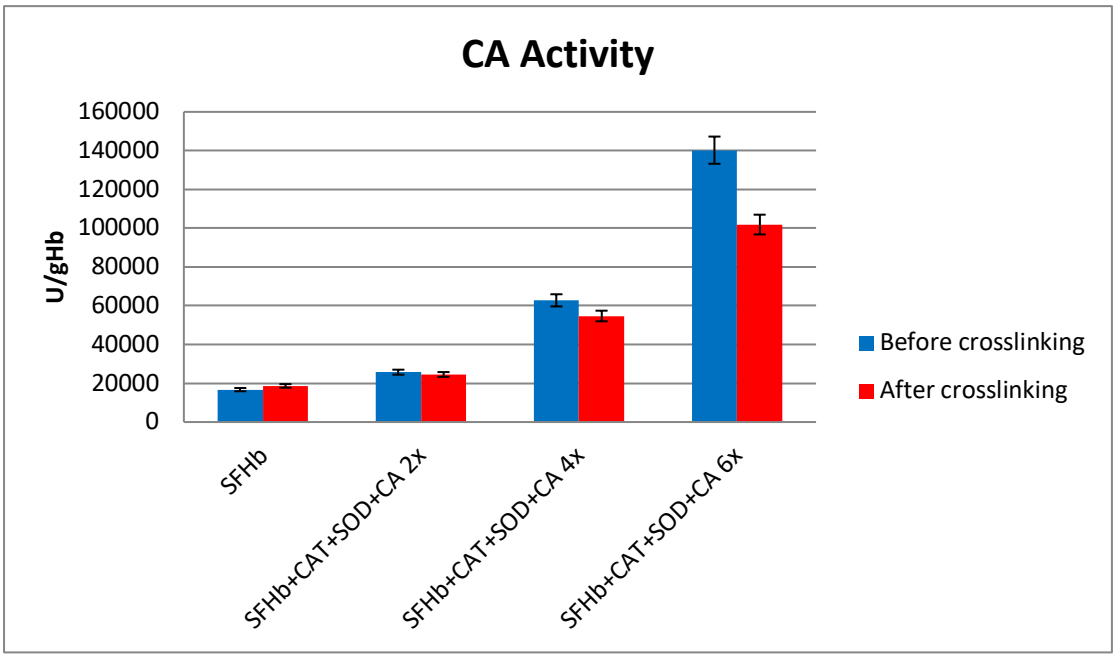


Figure 4: Comparison of CA activities in different samples. Left to right: SFHb before and after crosslinking; SFHb+CAT+SOD+CA 2x (SFHb plus 2x extracted SOD, CAT and CA) before and after crosslinking; SFHb+CAT+SOD+CA 4x (SFHb plus 4x extracted SOD, CAT and CA) before and after crosslinking; SFHb+CAT+SOD+CA 6x (SFHb plus 6x extracted SOD, CAT and CA) before and after crosslinking.

Discussion and Conclusion

Potassium phosphate buffer at pH 7.4 can prevent CAT from aggregation after ethanol-chloroform treatment. It is likely that the ion strength and pH value of the buffer make the

contributions in the aggregation of hemoglobin and the stability of three enzymes. The molecular weights (MW) of hemoglobin, CAT, SOD and CA are 68KDa, 250KDa, 32.5KDa and 29KDa respectively. The isoelectric points(pI) of hemoglobin, CAT, SOD and CA are 6.8, 5.4, 4.95 and 5.9 respectively. As we can see, only the pI of hemoglobin is close to the pH of solution at 7.4; thus hemoglobin tends to aggregate when adding organic solvents that have reduced dielectric strength[Crowell, Wall & Doucette, 2013]. In spite of its low pI value, CAT also aggregates after ethanol-chloroform, possibly due to its large size and the ion paring effect. However, when increasing the final concentration of phosphate ions from 20.3mM to 257.8mM, the improvement in CAT recovery yield can be observed (Table I). The phosphate buffer may be able to protect CAT because a large amount of ions migrate to the charged surface residues to generate a solvent layer to prevent the aggregation of CAT; while the pI of hemoglobin is close to the pH of solution, so hemoglobin cannot attract enough ions to form a hydration shield. Interestingly, the phosphate buffer was reported to destabilize the hemoglobin by binding to 2,3-bisphosphoglycerate (2,3-BPG) site [Ugwu&Apte , 2004].Therefore, the phosphate buffer at pH 7.4 can protect CAT and simultaneously promote hemoglobin precipitation.

Why is it that compared with the original assayed enzyme activities in SFHb, the apparent CAT activities increases in the extracted enzyme solution after ethanol-chloroform treatment. There are a number of possible explanations. Firstly, hemoglobin has peroxidase-like activity [Paco, et al, 2009]. We have done the following test on the effect of hemoglobin on CAT activity. When CAT is added to the SFHb, the total CAT activity of the mixture is lower than the summation of CAT activities in the extracted enzymes and the SFHb (Fig2). Therefore, it is likely that hemoglobin may have suppressed the CAT as a competitive inhibitor. Secondly, ethanol has been found to increase the CAT activity [Magner & Klibanov,1995]. We compared the activity of pure CAT and CAT-ethanol mixture. The ethanol concentration in this test is equal to the concentration during the extraction procedure. The result shows that 12.5% ethanol can increase CAT activity by 1.4 times. Thirdly, hemoglobin can be converted to choleglobin by coupling with ascorbic acid in the presence of oxygen [Miller 1958]. Choleglobin formation can even increase when red blood cells are hemolyzed [Foulkes& Lemberg,1949]. Hemoglobin-ascorbic acid complex is the intermediary substance in choleglobin formation and acts as a hydrogen peroxide donor [Miller 1958]. CAT assay is based on the measurement of H_2O_2 left after the reaction of samples withH_2O_2 soluti

In conclusion, we have devised a novel method to simultaneously extract all three enzymes, CAT, SOD and CA, from RBC hemolysate (SFHb). When using bovine RBC, the unlimitedto and inexpensive source of bovine blood would be much less costly than commercial enzymes for the scale up production of blood substitute. Earlier studies from this laboratory show that bovine PolySFHb containing the bovine red blood cell enzymes did not have immunological reactions when tested in rats (Zhu et al 2010). We shall investigate the effects of using higher concentrations of enzymes. If there is immunological problem, we can also use our novel method for the extraction of human hemoglobin and enzymes from human placental RBCs (cord RBCs) for the preparation of polySFHb-SOD-CAT-CA.

Reference

Alayash, A.I., *Redox biology of blood.* Antioxid Redox Signal, 2004. **6**(6): p. 941-3.

Arthurs, G. and M. Sudhakar, *Carbon dioxide transport.* Continuing Education in Anaesthesia, Critical Care & Pain, 2005. **5**(6): p. 207-210.

Bian, Y., Z. Rong, and T.M. Chang, *Polyhemoglobin-superoxide dismutase-catalase-carbonic anhydrase: a novel biotechnology-based blood substitute that transports both oxygen and carbon dioxide and also acts as an antioxidant.* Artif Cells Blood Substit Immobil Biotechnol,

2012. **40**(1-2): p. 28-37.
Bian, Y. and T.M.S. Chang, *A novel nanobiotherapeutic poly-[hemoglobin-superoxide dismutase-catalase-carbonic anhydrase] with no cardiac toxicity for the resuscitation of a rat model with 90 minutes of sustained severe hemorrhagic shock with loss of 2/3 blood volume.* Artificial cells, nanomedicine, and biotechnology,2015: **43**:p. 1-9.
Chang, T.M., *SEMIPERMEABLE MICROCAPSULES.*Science, 1964. **146**(3643): p. 524-5.
Chang, T.M., *Stabilisation of enzymes by microencapsulation with a concentrated protein solution or by microencapsulation followed by cross-linking with glutaraldehyde.* Biochem Biophys Res Commun, 1971. **44**(6): p. 1531-6.
Chang, T.M., Monograph on "ARTIFICIAL CELLS: biotechnology, nanotechnology, blood substitutes, regenerative medicine, bioencapsulation, cell/stem cell therapy" World Scientific Publisher/Imperial College Press 2007, 435 pages. http://www.medicine.mcgill.ca/artcell/2007%20ebook%20artcell%20web.pdf
Chang, T.M., *From artificial red blood cells, oxygen carriers, and oxygen therapeutics to artificial cells, nanomedicine, and beyond.* Artif Cells Blood Substit Immobil Biotechnol, 2012. **40**(3): p. 197-9.
Chang T.M. Opening chapter on "Artificial Cells that started Nanomedicine" in book on "Selected Topics in Nanomedgicine) (ed.TMS Chang) World Science Publisher/Imperial College Press. 2013 pp 1-21
Crowell, A.M., M.J. Wall, and A.A. Doucette, *Maximizing recovery of water-soluble proteins through acetone precipitation.* Analytica chimica acta, 2013. **796**: p. 48-54.
da Costa Ores, J., et al., *Purification of carbonic anhydrase from bovine erythrocytes and its application in the enzymic capture of carbon dioxide.* Chemosphere, 2012. **88**(2): p. 255-9.
D'Agnillo, F. and T.M. Chang, *Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties.* Nat Biotechnol, 1998. **16**(7): p. 667-71.
Foulkes, E. and R. Lemberg, *The formation of choleglobin and the role of catalase in the erythrocyte.* Proceedings of the Royal Society of London. Series B-Biological Sciences, 1949. **136**(884): p. 435-448.
Geers, C. and G. Gros, *Carbon dioxide transport and carbonic anhydrase in blood and muscle.* Physiol Rev, 2000. **80**(2): p. 681-715.
Gu, J. and T.M. Chang, *Extraction of erythrocyte enzymes for the preparation of polyhemoglobin-catalase-superoxide dismutase.* Artif Cells Blood Substit Immobil Biotechnol, 2009. **37**(2): p. 69-77.
Jahr, J.S., et al., *HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery.* J Trauma, 2008. **64**(6): p. 1484-97.
Kim, H.W. and A.G. Greenburg (editors) He*moglobin-Based Oxygen Carriers as Red Cell Substitutes and Oxygen Therapeutics*. Springer.2014
Magner, E. and A.M. Klibanov, *The oxidation of chiral alcohols catalyzed by catalase in organic solvents.* Biotechnology and bioengineering, 1995. **46**(2): p. 175-179.
Miller, H., *The relationship between catalase and haemoglobin in human blood.* Biochemical Journal, 1958. **68**(2): p. 275.
Moore, E.E., et al., *Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial.* J Am Coll Surg, 2009. **208**(1): p. 1-13.
Paco, L., et al., *Catalase–like activity of bovine met–hemoglobin: Interaction with the pseudo–catalytic peroxidation of anthracene traces in aqueous medium.* Biotechnology journal, 2009. **4**(10): p. 1460-1470.
Powanda, D.D. and T.M. Chang, *Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion.* Artif Cells Blood Substit Immobil Biotechnol, 2002.

30(1): p. 23-37.
Sims, C., et al., *Skeletal muscle acidosis correlates with the severity of blood volume loss during shock and resuscitation.* J Trauma, 2001. **51**(6): p. 1137-45; discussion 1145-6.
Ugwu, S.O. and S.P. Apte, *The effect of buffers on protein conformational stability.* Pharmaceutical Technology, 2004. **28**(3): p. 86-109.
Zhu, H., et al., *The immunological properties of stroma-free polyhemolysate containing catalase and superoxide dismutase activities prepared by polymerized bovine stroma-free hemolysate.* Artif Cells Blood Substit Immobil Biotechnol, 2010. **38**(2): p. 57-63.

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