Possibility of trypsin treated dog erythrocytes as universal blood: evaluation by FTIR microspectroscopy

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Abstract

A common canine erythrocyte antigen is DEA 1.1 but it is the most immunogenic. In this study, Fourier transformed infrared (FTIR) microspectroscopy was used to examine the alterations of membrane biomolecules in trypsin-treated DEA 1.1[+] canine erythrocytes. Production of canine DEA 1.1[-] erythrocytes was achieved by treating DEA 1.1^{+} red cells (5% cell suspension) with 1 mg/ml trypsin for 120 mins, without affecting the intracellular 2,3-diphosphoglycerate level. FTIR microspectroscopy was employed to study the effects of trypsin-treatment on canine trypsinized red cell membrane components. Production of canine DEA 1.1- erythrocytes was achieved without affecting the intracellular 2,3-diphosphoglycerate level. The FTIR microspectroscopy data suggested that the trypsin treated dog erythrocytes were possible for use as universal blood.

Introduction

Mammalian erythrocytes have their own specific blood group antigens. The blood groups characterize numerous antigens classified as polymorphic and species-specific, which are present on the surface of erythrocytes and are detected by antibodies (Reid and Westhoff, 2003). It is important to perform blood typing and crossmatching before a blood transfusion. The crossmatching determine the compatibility between the blood donor and the recipient in order to minimize the post-transfusion reaction. (Giger *et al.*, 1995; Tocci & Ewing, 2009).

The Dog Erythrocyte Antigen (DEA) system is the only blood group classification that is internationally recognized. The DEA system is composed of 7 canine blood types with typing sera availability for only five of those (DEA 1, DEA 3, DEA 4, DEA 5 and DEA 7) (Hohenhaus, 2004). All dog erythrocyte antigens (DEAs) can stimulate alloantibodies. DEA 1.1 is the most clinically important as it has high immunogenicity. It is important to perform blood typing and crossmatching prior to a blood transfusion to determine the compatibility between the blood donor and the recipient. This approach minimizes the frequency of reactions and their severity (Giger *et al.*, 1995; Tocci and Ewing, 2009).

DEA 1.1- blood can be used in both DEA 1.1- or DEA 1.1+ recipients but the latter can only donate blood to those with the same DEA. DEA 1.1 mismatches can cause life-threatening transfusion reactions in sensitized dogs (Giger *et al.*, 1995; Hohenhaus, 2004). Approximately 50% of dogs are DEA 1.1+; therefore, typing for this antigen before transfusion has been recommended (Hale, 1995; Hohenhaus, 2004). The practice of exclusively transfusing DEA 1.1- RBC products to DEA 1.1- dogs limits sensitization and the occurrence of acute hemolytic transfusion reactions (Giger *et al.*, 1995). Typing for other DEAs and other common RBC antigens has been difficult owing to the limited availability of typing reagents, cumbersome technology and difficulty in interpreting agglutination results (Giger *et al.*, 2005).

In dogs, the lack of alloantibodies that occur naturally and are clinically relevant may preclude the need for having extended type-specific blood available for a first transfusion; however, the risk of sensitization may have a long-term impact on patient management if additional transfusions are required (Kessler *et al.*, 2010).

In order to avoid transfusion reactions and hemolysis all blood donations are checked to confirm the blood group of the donor. In addition, a classical crossmatch procedure where red blood cells from the donor are mixed with plasma from the recipient ensures that they are compatible without agglutination or lysis occuring in vitro. DEA1.1 negative is considered to be universal and may be used for all RBC transfusions. Most dogs can receive the universal dog blood type, regardless of their own blood type. It is desirable and useful to remove the DEA1.1 antigen in order to produce universal blood and at the same time to avoid one of the most dangerous, and indeed the most common, fatal transfusion reactions (Hohenhaus, 2004).

Little is known about the biochemical structure of canine blood group antigens. Molecular weights have been determined for some blood group antigens and are listed later in the sections on individual blood groups. The glycolipids in the canine erythrocytes membrane have been determined and may ultimately be associated with a specific blood group (Hohenhaus, 2004). Using immunoprecipitation techniques, a monoclonal antibody to DEA 1.1 has been used to identify 2 membrane proteins of 50 and 200 kD (Andrews *et al.*, 1992).

FTIR spectroscopy has become a powerful tool for elucidating the biochemical changes of biomolecules. The infrared absorption spectra of biological molecules can give us information on the change of biochemical compounds of the samples normally known as the fingerprint of a molecule (Kačuraˊkovaˊ and Wilson, 2001).

IR absorption bands at frequencies of between 3000-2800 and 1780-1700 cm^{-1} can be used to monitor lipid phase transitions along with studies of other physical states (Lewis and McElhaney, 2007). FTIR spectroscopy also can provide a means of determining changes in diseased or damaged tissues in order to provide information at the molecular level. Membranes can be studied as model systems, as isolated preparations and as components of intact cells (Lewis and McElhaney, 2007). Fourier Transform infrared spectroscopy (FTIR) can be applied to study the membrane phase behavior of cells that are relevant for biomedical applications such as red blood cells and platelets. FTIR studies are minimally invasive and do not require labeling. FTIR has been applied to the qualitative and quantitative study of the molecular conformational order in models for biological membranes (Mantsch and McElhaney, 1991).

Studies on intact human erythrocytes have demonstrated the utility of FTIR spectroscopy as a unique biophysical technique for obtaining molecular conformation information from live cells (Moore *et al.*, 1991). In this study, FTIR microscopy was employed to examine alterations to membrane biomolecules of trypsin-treated DEA 1.1+ canine erythrocytes.

Materials and Methods

Blood samples and typing of blood group: Three dogs were housed indoors and maintained according to Kasetsart University's stray dog shelter project (Kasetsart University, Bangkok, Thailand). Blood was collected by leg venipuncture and placed into commercial potassium EDTA vials (Fushino, Japan). The dogs were considered healthy based on physical examination and complete blood counting (CBC). Erythrocytes were separated by sedimentation at 1,000 *g* for 10 mins at 4 °C and washed with normal saline for 3 times. The buffer coat was removed by aspiration after each wash. The packed red cells were diluted to 5% red cell suspension in normal saline for further experiments. All other chemicals were from Sigma (St. Louis, MO, USA). The Kasetsart University Committee with approved protocol number ACKU02056, and care was taken to comply with the 3R concept.

Blood typing (DEA 1.1+ and DEA 1.1-) was conducted using Blood Group determination Assay

RapidVet-H Canine DEA 1.1 kit (DMS Laboratories Inc., Flemington, NJ, USA).

Trypsin treatment: Trypsin (Sigma-Aldrich, USA) (1 mg/ml of phosphate-buffer saline (PBS)) was incubated with 1 ml of 5% canine red cells at 37 °C for 120 mins. All experiments were performed in duplicate. Then the erythrocytes were washed 3 times with normal saline before being adjusted to 5% red cells.

Determination of 2,3-diphosphoglycerate (2,3-DPG) concentration and Statistical analysis: 2,3-DPG concentrations of untreated (control) and trypsinized dog erythrocytes were determined using UV-test kit (Roche Diagnostics, Switzerland) according to the manufacturer's instructions.

   The presented results were the means ± standard deviation of three samples with duplicate trials. The effect of trypsin treatment on 2,3-DPG concentrations of dog erythrocytes was examined by Mann-Whitney test of Unpaired t test. Analyses were carried out using GraphPAD.

FTIR microspectroscopy: Untreated and trypsin-treated erythrocytes (5% cell suspension) were fixed with 4% formalin in 0.85% NaCl for 30 mins. Then erythrocytes were spotted on to Mirr IR low e-microscope slides (Kevey) and rinsed 3 times with distilled water. The slides were dried under vacuum and keep in a desiccator until subjected to FTIR microscopy.

   FTIR spectra were collected in a Bruker IR spectrometer (Tensor 27) coupled to an IR microscope (Hyperion 2000) with 36x magnification. The spectra were processed using Optics User Software version 6.5 (Bruker Optics GmbH, Ettlingen, Germany). Second derivatives and vector normalizations were manipulated in order to account for differences in sample thickness, minimize baseline variations and allow visual comparison. Data analysis was carried out in the spectral range from 3000-2800 cm^{-1} and 1800-850 cm^{-1}. Second derivatives were generated from the original spectra, and subsequently vector normalized for multivariate statistical analysis using Unscrambler 10 software (Camo, Norway) (Naumann, 2000).

   The spectra were pre-processed by calculating second derivatives (Savitsky-Golay with 9 point smoothing) in order to minimize the effects of variable baselines and allowing individual bands that were overlapping in the raw spectra to be readily visualized as separate minima bands. Spectra were then normalized to account for possible differences in sample thickness using the Extended Multiplicative Signal Correction (EMSC) method (Martens *et al*, 2003).

Cluster analysis: Principal component analysis (PCA) is a multivariate technique which is used to eliminate redundant information (Jackson, 2003). Cluster analysis was performed on second derivative spectra using a nine smoothing point Savitzky–Golay. Different segments of FTIR spectra were analyzed and Ward's minimum variance method (provided by OPUS software) was used for the cluster analysis.

Results

Trypsin treatment: Blood samples from the Kasetsart University stray dog shelter project (Kasetsart University, Bangkok, Thailand) DEA 1.1+ dogs were used for trypsin digestion. Digestion with 1 mg/ml trypsin for 120 mins did not cause any hemolysis. Blood typing is based on agglutinating reaction in which the reagent or antibody reacts with the erythrocytes of the test subject. The RapidVet-H Canine DEA 1.1 kit was used to classifying dogs as DEA 1.1 DEA 1.1+ or DEA 1.1-. Trypsinized canine erythrocytes gave negative results in the agglutination test for DEA 1.1 antigen (Figure 1).

	DEA 1.1 Positive control	DEA 1.1 Negative control	Result
Untreated dog erythrocyte (Control)	Heamagglutination	No heamagglutination	Heamagglutination DEA 1.1 Positive
trypsin treated dog erythrocyte	Heamagglutination	No heamagglutination	No heamagglutination DEA 1.1 Negative

Figure 1   Hemagglutination reaction of DEA 1.1+ trypsin-treated and untreated canine erythrocytes. RapidVet-H Canine DEA 1.1 kit is used to classifying dogs as DEA 1.1 DEA 1.1+ or DEA 1.1-.

Red cell 2,3-DPG status: The intracellular level of 2,3-DPG is a sensitive indicator of the metabolic state of erythrocytes and reflects their oxygen-carrying capacity (Rodriguez-Franco *et al*., 1994). The 2,3-DPG concentration of trypsinized erythrocytes was 6.19 ± 0.94 mM while the untreated erythrocytes was 6.03 ± 0.82 mM (Figure 2).

FTIR microspectroscopy analysis: FTIR microspectroscopy was used for elucidation of the structure, physical properties and interactions of carbohydrates. FTIR microspectroscopy is able to provide interpretive and experimental data of untreated and trypsinized erythrocytes. The second derivative of the original spectra was used to identify the peak frequencies of characteristic components. Amide I band is sensitive to protein secondary structure. FTIR spectroscopy uses Amide I band to study the process of protein alteration in vitro. According to distinctive hydrogen bonding environments for the altered secondary structure, shifts are observed in the frequency of the Amide I band (Susi and Byler, 1983). By studying protein structure in vitro, the effects of temperature, pH, solvent and protein conformation can be systematically examined (Miller *et al*., 2013). Trypsin-treatment of canine erythrocytes resulted in significant IR spectral changes from control cells, especially in the regions of amide I and II bands at 1648 cm^{-1} and 1540 cm^{-1} respectively (Figure 1a). The second derivative spectra at amide I region (1700-1600 cm^{-1}) and the lipid absorption region (3000-2800 cm^{-1}) were significantly changed in trypsinized red cell samples (Figure 1b). However, both types of erythrocytes showed

contributions of the CH$_3$-stretching vibrations from endogenous proteins, mostly hemoglobin at 2958, 2929, and 2869 cm^{-1} (Stoll *et al*., 2011). There was a small shoulder at the β-pleated sheet structures (1627 cm^{-1}) of trypsin-treated erythrocytes, which might be due to the absence of biomolecular components removed by the protease. The α-helix structure content (1648 cm^{-1}) (Arrondo *et al*., 1993) was significantly higher content in the treated erythrocytes.

Principal component analysis (PCA) and partial least squares (PLS) modeling (Geladi, 1998) were used to examine the variability of all FTIR spectra data sets acquired. IR spectra of trypsinized and control erythrocytes were clearly separated into two clusters (Figure 2a). PCA is a multivariate technique in which a number of related variables, several spectral data points are transformed into a smaller number of dimensions (Jackson, 2003). PCA analysis could discriminate between treated and untreated erythrocytes on a two-dimensional (PC1(55%) vs. PC2(25%)) scores plot (Figure 2b). The assignments for major bands observed in FTIR spectra of erythrocytes are shown in Table 1, in which IR spectra were from Figure 2a and associated PC1 and PC2 of Figure 3a.

FTIR spectra were collected in a Bruker IR spectrometer (Tensor 27) coupled to an IR microscope (Hyperion 2000) with 36x magnification. Spectra were processed using Opus 6.5 software (Bruker), the dendrogram of a cluster analysis based on the FTIR microscopic spectra of trypsinized and control erythrocytes based on second derivative of the representative spectra. The trypsinized erythrocytes (branch A) were well separated from control erythrocytes (branch B) (Figure 3).
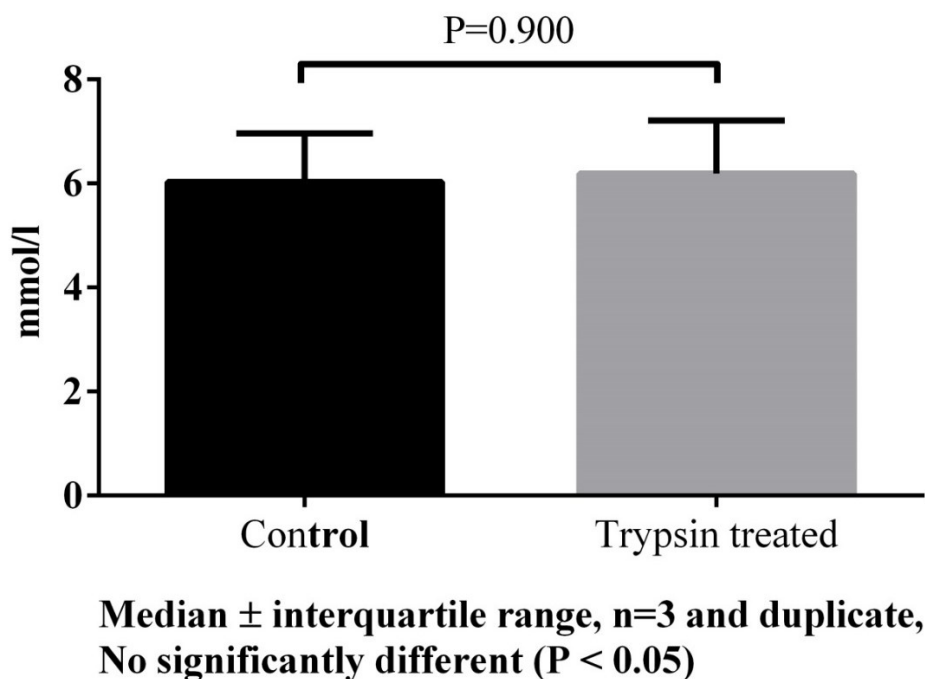
Figure 2   Level of 2,3-DPG determination from untreated dog erythrocytes and treated dog erythrocytes.

a)

b)
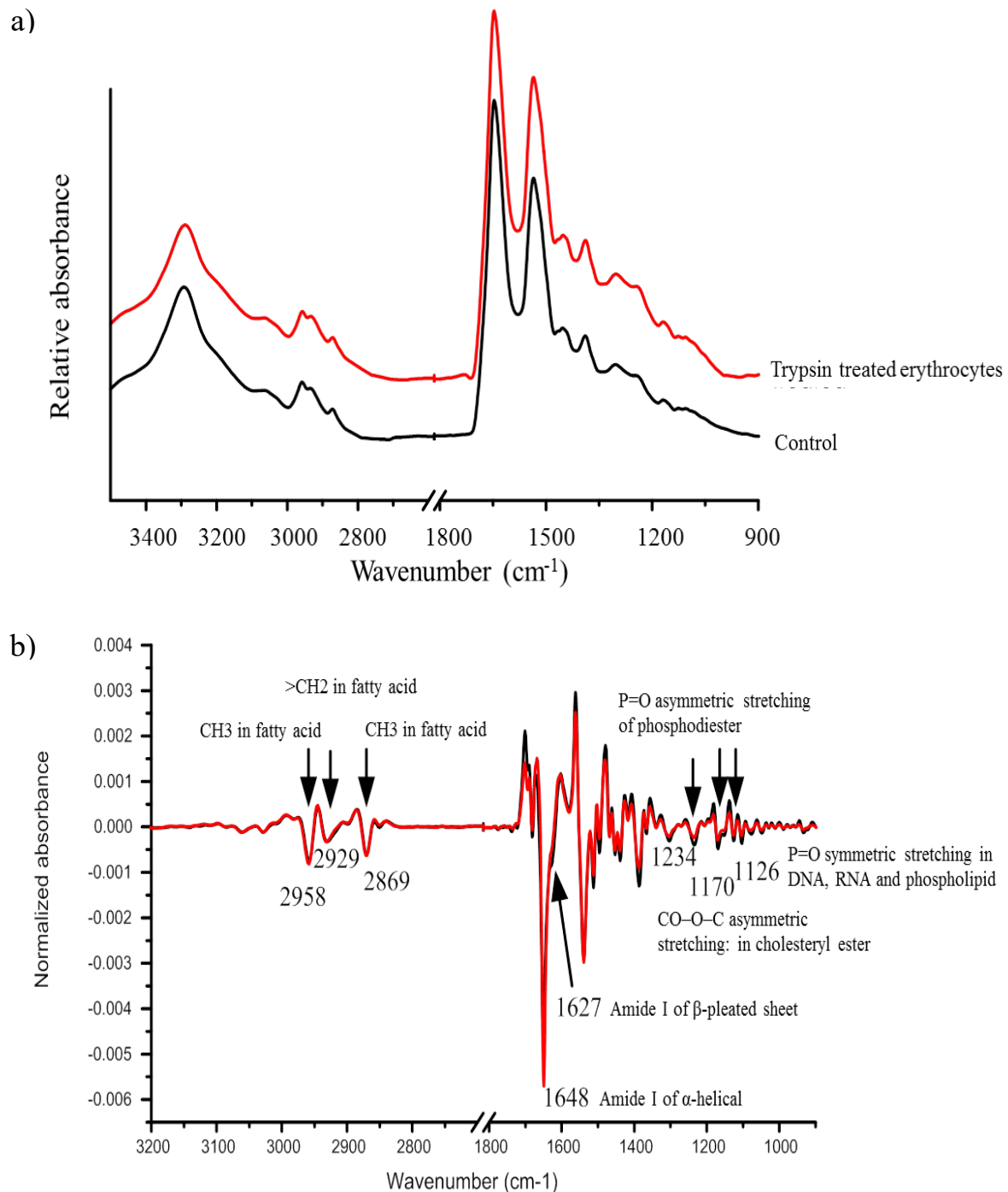
Figure 3 Representative FTIR spectra from untreated erythrocyte and trypsin-treated canine erythrocytes (a) and average second derivative FTIR spectra (b). Black line is control or untreated erythrocytes; red line is trypsin treated erythrocytes.

Discussion

To ensure that all erythrocytes were treated, all blood samples from DEA 1.1+ dogs were used for trypsin digestion with 1 mg/ml trypsin in water bath at 37 °C for 120 mins. After incubation, hemolysis had not appeared in any of the reaction tubes. The DEA 1.1+ dog erythrocytes were treated by trypsin to remove the extra membrane moieties especially blood group antigens. For Hemagglutination, the monoclonal antibody of RapidVet-H Canine DEA 1.1 kit reacted with the different amount of DEA 1.1 +

antigen. Therefore, strong, weak or missing reactions might occur. Positive control of RapidVet-H Canine DEA 1.1 kit showed strong reaction in the first row of untreated and treated dog erythrocytes. While, the DEA 1.1+ sample might have a lower number of antigen sites leading to weaker reaction. However, trypsinized dog erythrocytes gave negative results in the agglutination test for DEA 1.1 antigen by RapidVet-H Canine DEA 1.1 kit (Figure 1). The data showed that the DEA 1.1 antigen can be removed by trypsin,

indicating that DEA 1.1 is sensitive to proteolysis (under the conditions used).

The intracellular level of 2,3-DPG is a sensitive indicator of the metabolic state of erythrocytes and reflects their oxygen-carrying capacity (Rodriguez-Franco, et.al.,1994). From this study, 2,3-DPG concentration of the untreated erythrocytes (control) was 6.03 ± 0.82 mM while the normal level of 2,3-DPG from 93 healthy dogs' erythrocyte was 5.81 ± 0.07 mmol/L (Rodriguez-Franco, et.al.,1994). Trypsin treatment (1 mg/ml for 120 mins.) effectively converted canine DEA 1.1+ erythrocyte to DEA 1.1-, trypsinized erythrocytes 2,3-DPG concentration was 6.19 ± 0.94 mM, not significantly different from that of the control (6.03 ± 0.82 mM), data shown in Figure 2. This suggests that trypsin treatment (1 mg/ml for 120 mins.) was not adversely affecting the metabolic status of the dog erythrocytes, as judged by the absence of alteration in the intracellular 2,3-DPG level.

Fourier transform infrared (FTIR) spectroscopy is a technique that is sensitive to the secondary structure of proteins and has been widely used to investigate the process of misfolding and aggregate formation (Miller *et al.*, 2013; Miller *et al.*, 2013). The secondary structure of the intact and of the membrane-protected segments were compared for oriented membrane films by attenuated total-reflection Fourier-transform infrared spectroscopy and by circular dichroism and for vesicles suspension by circular dichroism and Raman spectroscopy. All the spectroscopic data indicated that the protease-resistant membrane-bound residue of the H1, K1-ATPase contains significant amount of β-sheet structure, both on films and in membrane suspensions. Polarized attenuated total-reflection infrared spectroscopy indicated that only the α-helical content of protease-resistant membrane-bound residue of the H1, K1-ATPase was oriented (parallel) with respect to the membrane normal. Raman spectroscopy revealed that Phe residues are preferentially removed by protease activity, suggesting that the membrane organization of the helices is independent of the part of the protein outside the membrane (Raussens, et.al., 1998b).

The secondary structure of the membrane part of the nicotinic receptor was analyzed by FTIR spectroscopy after removal of the receptor's extra membrane moieties by enzymatic proteolysis and showed that α-sheet structures are likely to be present in the membrane part of the protein (Görne-Tschelnokow, 1994). In this work, the assignments for major bands observed in FTIR spectra of erythrocytes are shown in Table 1. Principal component analysis (PCA) and partial least squares (PLS) modeling (Geladi, 1998) were used to examine the FTIR spectra data. IR spectra of trypsinized and control erythrocytes were clearly separated into two clusters (Figure 2a). PCA analysis could discriminate between treated and untreated erythrocytes on a two-dimensional (PC1 (55%) vs. PC2 (25%)) scores plot (Figure 2b). Proteolytic cleavage is efficient outside the membrane but does not reach the membrane part of the protein and the structure of the membrane part of the protein is not affected by the removal of the extramembrane loops (Raussens *et al.*, 1998b). Protease eliminates the part of protein residue which lies outside of the lipid bilayer on the cytoplasmic surface. These changes were more discernable when PCA was employed to generate two-dimensional scores plot (Corbalan-Garcia *et al.*, 1994). Therefore, FTIR spectromicroscopy showed that trypsin-mediated removal of the ectodomains of canine erythrocyte proteins and glycoproteins affected their secondary structures. In this experiment, DEA 1.1+ could be removed by trypsin with no agglutination with a monoclonal antibody against DEA1.1 (figure 1) and confirmed by the alteration of spectra from the FTIR spectromicroscopic technique (figure 3). However, this work focused only on the interaction between DEA1.1 antigen and trypsin. The proteolytic condition of blood group antigen and other proteins on erythrocytes should be further study for universal blood production. The possibility of trypsin treated dog erythrocytes as universal blood for transfusion should also be further study in clinical trial.

Table 1   Band assignments for mean IR spectra compared with the associated PC1 and PC2 regression coefficient loadings. IR spectra are from Figure 2a and associated PC1 and PC2 of Figure 3a.

Band maximum 2nd derivative spectra (cm-1)	Loading plots of PC1 and PC2 from PCA. (cm-1)	Band assignment
2958	2966	C-H asymmetric stretching of -CH3 in fatty acid
2929	2937	C-H asymmetric stretching of >CH2 in fatty acid
2869	2879	C-H symmetric stretching of -CH3 in fatty acid
	1699	C=O stretching in primary amide
1648	1660	Amide I of α-helical structures of protein
	1648	
1627	1621	Amide I of β-pleated sheet structures of protein
	1562	
	1477	
	1438	
	1386	
1234		P=O asymmetric stretching of phosphodiester in phospholipid
1170	1180	CO-O–C asymmetric stretching: ester bond in cholesteryl ester
	1182	
1126	1135	P=O symmetric stretching in DNA, RNA and phospholipid
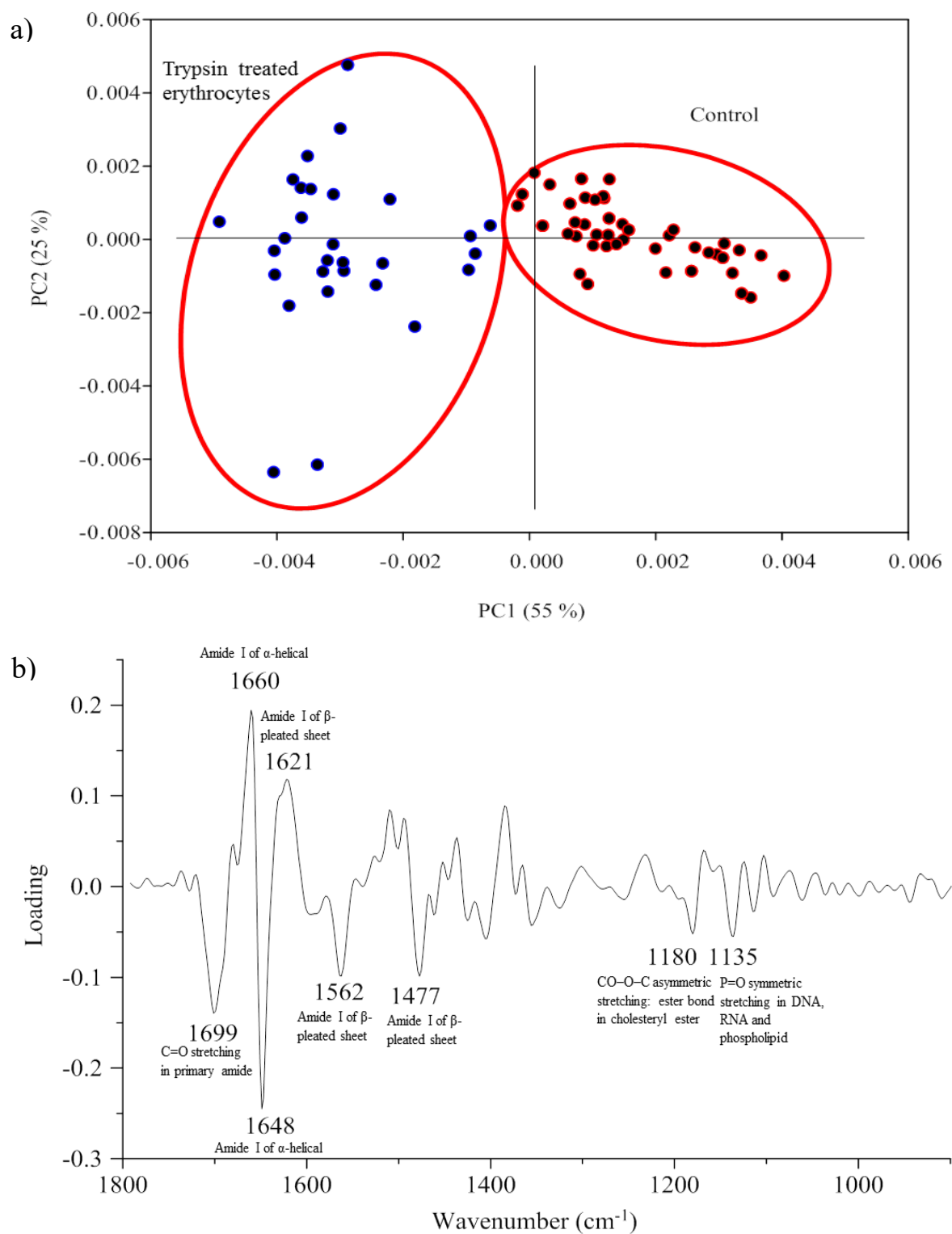
Figure 4   Principal component analysis (PCA) of second derivative FTIR spectra of untreated (control) and trypsin-treated canine erythrocytes (a) and loading plots of PC1 and PC2 from PCA.
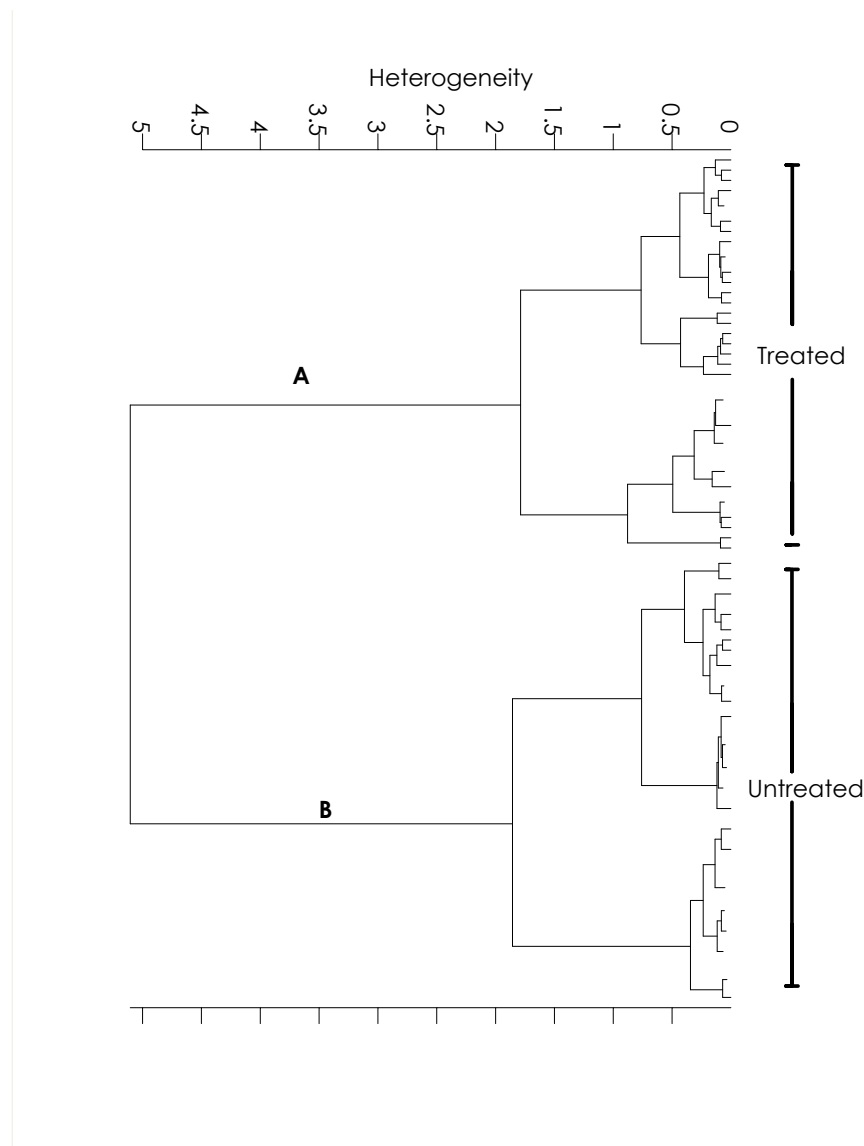
Figure 5 Dendrogram of a Hierarchical Cluster Analysis performed with FTIR spectra of untreated and trypsin-treated canine erythrocytes collected by FTIR microspectroscopy.
Dendogram was performed using vector normalized first derivative spectra. Cluster analysis using Ward's algorithm was performed on the second derivative of representative spectra.

In summary, this study has demonstrated the feasibility of creating a universal canine blood donor through protease removal of surface protein and/or glycoprotein antigenic determinants without affecting the metabolic status of the red cells. This may reduce acute hemolytic transfusion reaction resulting from DEA 1.1 incompatibility. Although the process did not appear to FTIR, spectromicroscopy indicated changes to the treated red cells' membrane bilayer and whether such perturbations will have a negative impact on potential use as a universal donor blood remains to be investigated further.

In conclusion, the FTIR microspectroscopy data suggested that the trypsin treated dog erythrocytes were possible for use as universal blood.

Acknowledgements

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