Enzymatic cleavage of cell surface proteins of pig and cow erythrocytes and its effect on concanavalin-mediated agglutinability

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Study was carried out to understand and compare architecture of the proteins of erythrocyte cell surface of some mammals viz., Homo sapiens (human), Sus scorfa domestica (pig) and Bos taurus domestica (cow). In this study, we investigated the action of proteinases viz., trypsin and chymotrypsin and neuraminidase on the erythrocyte surface proteins and erythrocyte agglutination tendency with a lectin (concanavalin A). The electrophoretic pattern of membrane proteins and glycoporphins (analyzed by SDS-PAGE and visualized by Coomassie brilliant blue and periodic acid-schiff stains, respectively) and concanavalin A (Con A) agglutinability revealed that: (i) There were variations in the number and molecular weights of glycoporphins in human, pig and cow, (ii) trypsin action on pig and cow erythrocyte membrane proteins was similar, unlike human, (iii) glycoporphins degradation by trypsin and chymotrypsin was not similar in pig, as compared to that of human and cow, (iv) erythrocytes agglutination with Con A was significantly different due to differences in membrane composition and alterations in the surface proteins after enzyme treatment, (v) a direct correlation was found between degradation of glycoporphins and Con A agglutinability, and (vi) removal of erythrocyte surface sialic acids by neuraminidase specifically indicated an increase in Con A agglutinability of pig and cow erythrocytes, similar to human.

Keywords: Agglutination, Mammalian erythrocyte, Membrane proteins, Glycophorins, Proteinases, Neuraminidase

The selective action(s) of proteinase and neuraminidase on the external surface of the intact erythrocyte of human has been used extensively in topological analysis of the erythrocyte membrane. The membrane’s surface architecture can thus be described in terms of proteinase and neuraminidase cleavage sites located within the external domains of susceptible membrane polypeptides. Conclusive evidence about proteinase and neuraminidase action(s) on the intact erythrocyte surface and its impact on lectin-mediated agglutinability of erythrocytes of other mammalian species is still lacking. Enzyme-digestion sites are reported within the external domains of human erythrocyte membrane proteins1-5. The chymotrypsin digestion at the external face of intact erythrocytes generates a carboxyl-terminal fragment of the band 3 molecule6. Trypsin causes digestion of PAS-1 (glycophorin A dimer) and PAS-2 (glycophorin A monomer) bands. Glycophorins are sialoglycoproteins (sialic acid rich glycoproteins) and neuraminidase treatment of erythrocyte surface releases N-acetyl neuraminic acid (sialic acid) from glycophorins and decreases the cell surface charge7. Proteinases also modify several membrane proteins of other non-human mammalian erythrocytes8-10. As a consequence of modifications occurring in the membrane, cells acquire a high agglutinability with plant lectins11,12.

The detailed studies have been performed to elucidate the basis of the lectin-agglutinability and several factors that influence the agglutinability of cells have been identified13-15. The major factors responsible for agglutination are: i) cell-surface charge due to sialic acid residues, ii) number and properties of the lectin receptors, iii) mobility of the lectin-receptor complexes in the membrane, iv) the mobility-modulating agents, such as cytoskeletal elements, and v) cellular deformability.

The considerable information is available on the concanavalin A (Con A)-mediated agglutination of human erythrocytes after treatment with proteolytic enzymes2,13,16,17, as do normal nucleated cells18. The glycophorins, especially glycophorin A of erythrocytes due to presence of rich sialic acid appears to be responsible for inhibition of agglutination with Con A. Thus, human erythrocytes provide detailed information of the possible factors
which affect agglutination. Band 3 has been identified as Con A receptor in human erythrocytes. Treatment with trypsin and neuraminidase causes the agglutination of rabbit erythrocytes, but has almost no effect on sheep erythrocytes. Possible role of cell surface charge and zeta potential in determining Con A-agglutinability of erythrocytes of animals like sheep and rabbit has been reported.

In the present study, we have investigated the action(s) of proteinases and neuraminidase on the erythrocyte agglutination by Con A of two economically important mammals (pig and cow), as compared to human erythrocytes.

Materials and Methods

Coomassie brilliant blue R-250, concanavalin A (IV), TLCK-chymotrypsin, N, N'-methylene bisacrylamide, neuraminidase (type VI from *Clostridium perfringens*), N,N,N',N'-tetramethylmethylenediamine, phenyl methyl sulfonylfluoride, sodium dodecyl sulfate and TPCK-trypsin were obtained from Sigma Chemicals Co., St. Louis, MO, USA. All other biochemicals used were of the grade suitable for gel electrophoresis and PAS staining.

Isolation of erythrocytes

Human blood was obtained from healthy donors at Devi Ahilya University, Indore (MP), India. Pig and cow blood samples of healthy animals were obtained from local slaughterhouse and Veterinary Hospital, respectively. All blood samples were collected in acid/citrate/dextrose (anticoagulant) solution. Erythrocytes were obtained by removing plasma and buffy coat from blood by centrifugation at 1000 × g for 5 min at room temperature (RT) and further washed with 10 vols. of cold Tris buffer saline (TBS - 0.01 M Tris-HCl buffer, pH 7.4 with 150 mM NaCl) four-times at same g value.

Enzyme treatment of erythrocytes

Washed erythrocytes were suspended in two vols. of proteinase solution (trypsin or chymotrypsin) and incubated at 37°C for 90 min. Trypsin (100 µg/ml) and chymotrypsin (250 µg/ml) were prepared in TBS. Neuraminidase was dissolved in 0.1 M Tris-maleate buffer containing 0.11 M NaCl, pH 5.6. After incubation, the cells were washed four-times with at least ten vols of chilled TBS.

Isolation of membranes

Membranes were prepared from normal and enzyme-treated erythrocytes according to Hanahan and Ekholn. Washed erythrocytes were lysed by mixing with 30 vols. of cold 0.01 M Tris-HCl buffer, pH 7.4 with 1 mM phenyl methyl sulphonyl fluoride (PMSF). After 15 min in cold, the suspension was centrifuged at 22,000 x g for 15 min in a refrigerated centrifuge at 4°C. The resulting deep red supernatant was discarded. The small opaque button seen below the translucent pellet of membranes was carefully removed. The membranes were suspended in 0.01 M Tris-HCl buffer, pH 7.4 and recentrifuged. In this way, the membranes were washed four-times, until a milky white preparation was obtained. In the last washing, 0.05% NaN₃ was added to the washing buffer to prevent microbial growth.

Separation of membrane proteins

The protein in different erythrocyte membrane preparations was estimated. Membrane protein separation by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed according to Laemmli with some modifications. The slab gel (1.5 mm thickness) consisted of 10% acrylamide in running gel (pH 8.8) and 5% acrylamide in stacking gel (pH 6.8). Protein samples were solubilized in sample buffer containing 0.031 M Tris, 0.25% β-mercaptoethanol and 5% glycerol in final volume and heated at 100°C for 10 min before loading on to the gel. Electrophoresis was carried out at constant current of 2 mA/cm using electrode buffer (0.025 M Tris, 0.2 M glycine, 0.2% SDS).

After electrophoresis, gels were removed from the glass plates and processed further for staining and destaining in a washed plastic container.

Coomassie brilliant blue (CBB) staining

Before CBB staining, the gels were fixed for 30 min in 40% methanol-10% acetic acid and then stained overnight in the same solution containing 0.1% (w/v) CBB. Gels were destained in 40% methanol-10% acetic acid.

Periodic acid-Schiff’s (PAS) staining

The carbohydrate-specific staining was performed to visualize sialoglycoproteins. SDS was removed from the gel by stirring in solution containing 25% isopropanol, 10% acetic acid for 12 h, followed by washing with 10% acetic acid for 10 h. The gel was then sequentially treated with staining reagents with gentle stirring at RT containing 0.5% periodic acid for 2 h; 0.5% sodium arsenite and 5% acetic acid for 30 min, 0.1% sodium arsenite and 5% acetic acid for 2 min, 5% acetic acid for 20 min and Schiff reagent
for overnight. The gel was destained in 0.1% sodium metabisulphite in 0.01 N HCl for several hours with intermittent changes till the background became clear.

Restaining of PAS-stained gels with CBB
PAS-stained gels were restained in 0.01% CBB stain for 30 min as given above.

Gel image analysis
The CBB and PAS stained gels were scanned using Scanner ‘HP scanjet 7400c’. Densitograms of the gel images were obtained by using the UVP BiolImaging Systems “LabWorksTM Image Acquisition and Analysis software, Version 4.0.0.8”.

Agglutination assay of untreated and enzyme-treated erythrocytes
A 0.4% (v/v) suspension of untreated (normal) or enzyme-treated erythrocytes was mixed with an equal volume of the freshly prepared Con A solution in TBS at RT. A control containing the relevant inhibitory sugar (α-methyl D-mannopyranoside, 0.05 M) and the highest Con A concentration (250 µg/ml) was used in the assay. After 45 min incubation at 37°C, the tubes were tapped several times and unagglutinated cells (free or in two-celled aggregates) were counted using a hemocytometer under the microscope. The extent of agglutination was calculated as described by Gokhale and Mehta2 as follows:

100 - (100 × number of unagglutinated cells) / number of input cells

Alternatively, the same assay was performed using microtitre plate and agglutination was judged by visual appearance of erythrocyte pellets.

Statistical analysis
The agglutination data were expressed as mean ± standard error (S.E.M.) and analyzed by one-way analysis of variance (ANOVA), followed by a post-hoc Newman-Keuls multiple comparison test using a trial version of PRISM 5 software for windows (GraphPad Software, Inc., La Jolla, CA, USA). For statistical evaluation of results, significance was defined by a probability level of p < 0.05. All the experiments were carried out five-times.

Results
Proteolytic action of proteinases on mammalian erythrocyte membrane proteins
To understand proteolytic action of trypsin and chymotrypsin on mammalian erythrocyte membrane proteins exposed to cell surface, the intact erythrocytes were subjected to proteolytic digestion by treatment with both trypsin and chymotrypsin. The membranes were isolated from extensively washed proteinase-treated erythrocytes and analyzed by SDS-PAGE, followed by CBB staining (Fig. 1). The human erythrocyte membranes protein profiling of untreated (lane 1-H) and trypsin (lane 2-H) treated erythrocytes was almost similar, indicating no action of trypsin on erythrocyte surface proteins. The action of chymotrypsin on band 3 was indicated by the appearance of an intense band of Mr 58 kDa2,25. The densitometric analysis (Fig. 1, DS-H) indicated the reduction in peak intensity of band 3 and a sharp increase near the position of band 4.5, Mr 58 kDa.

The results of digestion of pig erythrocytes with proteinases indicated the action of both trypsin and chymotrypsin, but the extent of action was not significant as revealed by SDS-PAGE, followed by CBB staining (Fig. 1, lanes 2-P, 3-P). Proteinases acted on both band 3 and 4.5. Band 4.5 was extensively digested by both the proteinases, but a very less action was seen on band 3. The pig erythrocytes membrane surface proteins were equally susceptible to both the proteinases, showing actions on similar proteins (Fig. 1, lanes 2-P, 3-P).

In case of cow, both proteinases acted sharply on band 3 (Fig. 1, lanes 2-C, 3-C). The degraded fragments of 75 and 65 kDa were found after the action of trypsin and chymotrypsin (Fig. 1, lanes 2-C and 3-C). Band 3 degradation of cow erythrocyte by proteinases was more, as compared to human erythrocyte membrane; the degradation products were also of higher Mr than human erythrocyte membrane. The densitogram (Fig. 1, DS-C) showed the action of both proteinases on band 3, as revealed by the low level of peak (lane 3) at the position of band 3 and a sharp high peak just above the position of band 4.5.

Action of proteinases on mammalian erythrocyte membrane glycophorins
The proteolytic action of trypsin and chymotrypsin on mammalian erythrocyte membrane glycophorins was studied by subjecting the SDS-Polyacrylamide gel to sialoglycoprotein-specific PAS stain24 (Fig. 2). In trypsin-treated human erythrocytes membrane glycophorins, PAS 1 and PAS 2 were found to be degraded to a higher extent (Fig. 2, lane 2-H). A new fragment of 62 kDa (marked by green arrow) was observed, indicating that the trypsin degradation product of PAS bands retained in the membrane2. In
chymotrypsin-treated human erythrocytes (lane 3-H), a decrease in intensity of PAS-1 and PAS-2 was observed.

In case of pig erythrocytes (Fig. 2), trypsin did act to a lesser extent on all the PAS bands, as compared to human erythrocytes (lane 2-P). The degradation products of these glycophorins were observed in the region of PAS-PIV (36 kDa). In case of chymotrypsin (lane 3-P), all glycophorins (PAS-PI to PAS-PIII) were cleaved, except PAS-IV and degradation products had same Mr as that of trypsin degradation products. Densitogram (Fig. 2, DS-P) also showed changes in the peak intensities after proteinase action.

In cow erythrocytes, action of trypsin (Fig. 2, lane 2-C) was observed on all glycophorins (PAS-CI to PAS-CVIII), except PAS-CV and a new degradation product (45 kDa) was observed in between the PAS-CV and PAS-CVI. The chymotrypsin (lane 3) action was observed on all the glycophorins, but was lesser as compared to trypsin. The degradation product (45 kDa) showed the same position on gel as in case of trypsin. The most intense glycophorin PAS-CI was partially digested by chymotrypsin. These findings were also confirmed in densitogram (Fig. 2, DS-C). The peak intensity in PAS-CI region was decreased to baseline and 50% in case of trypsin and chymotrypsin actions, respectively.

To find out the relative position of proteins, glycophorins and their degradation products formed due to action of proteinases on erythrocytes, the PAS-stained gel was restained with CBB stain (Fig. 3). This double-stained gel permits direct visualization of the position of glycophorins in relation with all other membrane proteins. In trypsin-treated human erythrocyte membranes (Fig. 3, lane 2-H), a new PAS-stained glycophorin fragment (62 kDa) was observed.
observed in the region of band 4.5. The new band 3 fragment generated due to chymotrypsin action was found just below the band PAS-4.

The restaining of PAS-stained gel with CBB of pig erythrocyte membrane treated with proteinases showed some lightly stained bands, indicating the relative position of new fragments of glycophorins at the position of band 7 (32 kDa) (Fig. 3, lanes 2-P, 3-P). The proteinase-treated cow erythrocyte membrane proteins stained with PAS were restained with light CBB stain. This dual stain indicated the formation of erythrocyte glycophorin fragments of 48 kDa size near band 4.9 (Fig. 3, lanes 2-C, 3-C).

Neuraminidase action on erythrocyte membrane glycophorins

Neuraminidase selectively acts on sialoglycoproteins by hydrolyzing terminal sialic acid (N-acetyl neuraminic acid) residues and such an action could be analyzed by sialoglycoprotein-specific PAS stain. Due to the action of neuraminidase on erythrocyte glycophorins, the removal of sialic acid was indicated by shifting of PAS bands on the polyacrylamide gel (Fig. 4). Action of neuraminidase on all the PAS bands of human erythrocyte membrane was observed as revealed by appearance of new fuzzy bands of Mr 81 kDa, 35 kDa and 22 kDa (Fig 4, lane 2-H) as reported earlier. Alterations in the position and intensities of PAS bands were very well depicted in the densitogram (Fig. 4, DS-H).

In pig erythrocyte membranes, PAS-PII and PAS-PIV were digested more by neuraminidase, as compared to PAS-PI and PAS-PIII (Fig. 4, lane 2-P). The densitogram (Fig. 4, DS-P) also indicated the neuraminidase action, as decreased peak intensities were observed. In case of cow erythrocyte membranes, neuraminidase action (Fig. 4, lane 2-C) was observed on all the glycophorin bands. PAS-CI and PAS-CIII bands showed upward shifts in their positions, while PAS-CII and PAS-CVII bands were...
extensively degraded by the neuraminidase. PAS-CIV to PAS-CVI glycophorin bands showed decreased intensity on the gel. The action on PAS-CVIII was not clearly visible because of very less intensity of that band on the gel.

Figure 4 (DS-C) shows densitogram having the pattern of untreated and neuraminidase-treated erythrocyte membranes. The peaks of PAS-Cl and PAS-CIII were shifted after the enzyme treatment, while all other peaks showed the same results as observed on the gel. Relative position of erythrocyte proteins and glycophorins after neuraminidase treatment on same gel was determined by restaining the PAS stained gel with CBB stain (Fig. 5).

Con A-mediated agglutinability of mammalian erythrocytes

Con A possesses binding affinity for band 3 on the erythrocyte surface, but not able to agglutinate native (untreated) human erythrocytes. Only after treatment with a proteolytic enzyme or neuraminidase, the cells become agglutinable with Con A. Con A-mediated agglutinability of mammalian erythrocytes after trypsin, chymotrypsin and neuraminidase treatment of erythrocytes was analyzed under microscope and also by microtitre plate assay. In microtitre plate assay (Fig. 7), 0C (zero control) in the first row had no Con A, while SC (sugar control) in the fourth row contained Con A (100 µg/ml) with inhibitory sugar (α-methyl D-mannopyranoside). The microtitre plate wells in second and third rows contained Con A 50 and 100 µg/ml, respectively. The microtitre plate assay (Fig. 7) showed results similar to obtained from microscopic agglutination assay. All animal erythrocytes did not show any agglutination in zero and sugar controls. The mammalian erythrocytes included in this study agglutinated after trypsin (100 µg/ml of enzyme solution), chymotrypsin (250 µg/ml of enzyme solution) and neuraminidase (0.01 unit/ml of enzyme solution) treatment.

A remarkable variation was observed in the extent of agglutination of mammalian erythrocytes (Figs 6 and 7). Extent of agglutination of trypsinized (Tr) erythrocytes at 50 µg/ml Con A concentration was less in pig (PTr, 18%) and cow (CTr, 46%), as compared to human (HTr, 62%) (Figs 6 and 7). The percent agglutination of trypsinized erythrocytes increased at 100 µg/ml Con A concentration for pig (54%) and cow (66%), as compared to human (85%).

Agglutination behavior of erythrocytes of all the animals differed significantly (p< 0.05) for trypsinized erythrocytes. The agglutination pattern of Tr-treated erythrocytes obtained from percentage agglutination formula as well as microtitre plate assay was as follows: Human > Cow > Pig. Extent of agglutination of chymotrypsinized (CTr) erythrocytes was higher in pig (PCTr) and cow (CCTr), as compared to human (HCTr) (Figs 6 and 7). The
Fig. 4—Analysis of membrane glycophorins obtained from neuraminidase treated erythrocytes by SDS-PAGE (10% Gel) followed by PAS stain [Lane 1: Untreated membranes (180 µg protein in each lane); lane 2: Neuraminidase-treated membranes (180 µg protein in each lane). DS: Densitogram; H: human; P: pig; C: cow. Black and green arrows indicate the proteins acted upon by neuraminidase and the new fragments generated after its action, respectively]

Fig. 5—Analysis of membrane proteins and glycophorins obtained from neuraminidase treated erythrocytes by SDS-PAGE (10% Gel), followed by restaining of PAS stained gel with CBB stain [Lane 1: Untreated membranes (180 µg protein in each lane); lane 2: Neuraminidase-treated membranes (180 µg protein in each lane). H: human; P: pig; C: cow. Black and green arrows indicate the protein acted upon by neuraminidase and the new fragments generated after its action, respectively]
values of percent agglutination in chymotrypsinized (CTr) erythrocytes of pig and cow at 50 µg/ml of Con A concentration were 94 and 76%, as compared to human (39%). The percentage agglutination of CTr erythrocytes of pig and cow at 100 µg/ml of Con A concentration was 97 and 83% respectively, as compared to human (54%). The agglutination behavior of CTr erythrocytes of all the animals differed significantly (p<0.05). PCTr and CCTr showed higher agglutination, as compared to HCTr erythrocytes. Agglutination pattern of chymotrypsin-treated erythrocytes obtained from percentage agglutination formula as well as microtitre plate assay was as follows: Pig > Cow > Human.

Extent of agglutination in chymotrypsinized (CTr) erythrocytes of pig and cow at 50 µg/ml of Con A concentration was 94 and 76%, as compared to human (39%). The percentage agglutination of CTr erythrocytes of pig and cow at 100 µg/ml of Con A concentration was 97 and 83% respectively, as compared to human (54%). The agglutination behavior of CTr erythrocytes of all the animals differed significantly (p<0.05). PCTr and CCTr showed higher agglutination, as compared to HCTr erythrocytes. Agglutination pattern of chymotrypsin-treated erythrocytes obtained from percentage agglutination formula as well as microtitre plate assay was as follows: Pig > Cow > Human.

of Con A. Agglutination pattern of Nr-treated erythrocytes obtained from percentage agglutination formula as well as microtitre plate assay was as follows: Human > Pig > Cow.

Discussion

Extensive literature is available on human erythrocyte membrane with respect to its structure and organization. Over 250 blood group determinants are known and most of these are located on integral erythrocyte membrane proteins and glycoproteins. The function of some of these membrane structures is known. Glycoproteins of human erythrocytes are reported to act as receptors for parasites. Cell surface features are also studied with respect to the action of proteinases and neuraminidase, followed by lectin agglutinability of human erythrocytes. There are few reports available related to the structural features of erythrocyte proteins and glycoporphins of non-human mammals.

In this study, we investigated structural features of membrane proteins and glycoporphins of pig and cow, in comparison with human erythrocytes. However, it is reported that glycoporphins of erythrocyte...
membranes of pig, cow and human show marked differences in their number and size when visualized by sialoglycoprotein-specific PAS stain\textsuperscript{34}. Sialic acid residues of glycoporphins exposed to exterior face contribute to the cell surface charge of erythrocytes and any change in sialic acid content revealed by PAS-stained gels might be responsible for differences in the cell surface charge among these species, similar to human erythrocytes\textsuperscript{7}.

The action of proteinases \textit{viz.} trypsin and chymotrypsin on pig and cow erythrocytes, followed by analysis of their membranes by SDS-PAGE (stained with CBB) indicated that trypsin action on cow erythrocytes was more, especially on band 3 as compared to pig erythrocytes (Fig. 1). Cow erythrocyte membrane band 3 appeared to have action sites for both the proteinases. It seemed that cow erythrocyte had topology of band 3 for chymotrypsin action, similar to human. Trypsin had no action on human erythrocyte membranes proteins, as indicated by CBB stained gel. Trypsin-treated pig and cow erythrocyte membranes showed the presence of some new fragments. This indicated that possibly some lysine and/or arginine residues of proteins were exposed on cell surface for trypsin action in pig and cow that were not available in case of human erythrocytes.

Similarly, chymotrypsin exhibited action on aromatic/hydrophobic amino acid residues of human and cow erythrocytes, but not to that extent on pig erythrocyte surface proteins. There are reports about the action of chymotrypsin on band 3 of human erythrocytes exposed to cell surface\textsuperscript{6}. Action of proteinases on glycoporphins, represented by PAS bands, of human erythrocyte membrane was in agreement with previous literature\textsuperscript{1,2}. Trypsin showed action on PAS 1 and PAS 2 bands of human and PAS-CI to CIV bands of cow erythrocytes. On the other hand, chymotrypsin showed partial or no action on these glycoporphins. In case of pig erythrocytes, trypsin and chymotrypsin both exhibited action on glycoporphins PAS PI to PIV. Thus, trypsin showed a partial, while chymotrypsin a drastic action on pig erythrocyte glycoporphins. The Nr-treated pig and cow erythrocyte membranes showed decrease in PAS band intensities or change in their positions on the gel due to the removal of sialic acid residues. This alteration in PAS banding pattern suggested the action of neuraminidase on sialic acid residues of glycoporphins exposed to cell surface, similar to human erythrocytes\textsuperscript{7}.

The mammalian erythrocytes became agglutinable with Con A after treatment with proteinases or neuraminidase. The action of proteinases on band 3 did not correlate with their ability to increase the Con A agglutinability, as chymotrypsin-treated human erythrocytes (showing marked action on band 3) had minimum agglutination among these three species. On the other hand, chymotrypsinized pig erythrocyte had very less action on band 3 and showed maximum agglutination with Con A. The ability of Con A to cause the agglutination was possibly related to the extent of degradation of glycoporphins, in particular PAS bands PII and PIII of pig and CI and CVIII of cow erythrocytes. As indicated by PAS stained gels, a direct correlation was found between degradation of glycoporphins and Con A agglutinability similar to human erythrocytes\textsuperscript{2,7}.

Action of neuraminidase specifically demonstrated the negative role of cell surface sialic acids on Con A agglutinability, similar to human erythrocytes. Since glycoporphins possess a very large proportion of total sialic acids of erythrocyte membrane, their removal by neuraminidase would decrease the overall surface charge and might facilitate the approach and interaction between the cells during agglutination. As Con A has shown maximum affinity for binding with mannose and glucose\textsuperscript{35}, they determine the number of Con A binding sites on the erythrocyte cell surface. Thus, increase of agglutination might be due to the exposure of such Con A binding sites that were hidden prior to the enzyme treatments.

In conclusion, present studies clearly indicated some basic differences in human, pig and cow erythrocyte membrane proteins, especially with respect to glycoporphins that determined the Con A-mediated agglutinability after enzyme treatment of erythrocytes.

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