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Differential actions of proteinases and neuraminidase on mammalian erythrocyte surface and its impact on erythrocyte agglutination by concanavalin A

Savita Sharma and Sadashiv M. Gokhale

School of Biochemistry, Devi Ahilya University, Khandwa Road, Indore 452017, India

Abstract. Action of proteinases *viz.* trypsin and chymotrypsin, and neuraminidase on intact erythrocyte membrane proteins and glycophorins (sialoglycoproteins) exposed to cell surface and its impact on lectin (concanavalin A)-mediated agglutination were studied in *Homo sapiens* (human), *Capra aegagrus hircus* (goat) and *Bubalus bubalis* (buffalo). Membrane proteins and glycophorins analysis by SDS-PAGE as visualized by coomassie brilliant blue and periodic acid-schiff stains, respectively, and agglutination behaviour revealed marked differences: 1) there were prominent dissimilarities in the number and molecular weights of glycophorins in human, goat and buffalo erythrocyte membranes; 2) proteinase action(s) on human and buffalo erythrocyte surface membrane proteins and glycophorins showed similarity but was found different in goat; 3) significant differences in erythrocyte agglutinability with concanavalin A can be attributed to differences in membrane composition and alterations in the surface proteins after enzyme treatment; 4) a direct correlation was found between degradation of glycophorins and concanavalin A agglutinability; 5) action of neuraminidase specifically indicated the negative role of cell surface sialic acids in determining concanavalin A agglutinability of goat and buffalo erythrocytes, similar to human. Present studies clearly indicate that there are some basic differences in human, goat and buffalo erythrocyte membrane proteins, especially with respect to glycophorins, which determine the concanavalin A-mediated agglutination in enzyme treated erythrocytes.

Key words: Erythrocyte membrane proteins — Glycophorins — Proteinases — Neuraminidase — Agglutination

Introduction

The human erythrocyte membrane surface has proteinase-cleavage sites located within the external domain of susceptible membrane polypeptides (Dzandu et al. 1985; Gokhale and Mehta 1987a; Hamasaki et al. 1997; Ali and Tayyab 2001; Rashid et al. 2001). A carboxyl-terminal fragment of the band 3 molecule is generated by digestion with chymotrypsin at the external face of intact erythrocytes (Markowitz and Marchesi 1981) and the cleaved fragments are retained within the membrane, while trypsin has no action on erythrocyte membrane proteins. It causes digestion of glycophorins *viz.* PAS-1 (glycophorin A dimer) and PAS-2 (glycophorin A monomer) bands. Glycophorins are sialoglycoproteins (sialic acid rich glycoproteins) and neuraminidase releases sialic acid (N-acetyl neuraminic acid) from glycophorins which decreases the cell surface charge (Gahmberg and Andersson 1982). Proteinases also modify several membrane proteins of bovine, equine and porcine erythrocytes (Makino et al. 1984; Moriyama et al. 1994; Okamura et al. 2007).

As a consequence of alterations occurring in the membrane, cells acquire a high agglutinability with plant lectins (Abu et al. 1963; Anderson et al. 2002). Extensive studies have been undertaken to elucidate the basis of the high lectin-agglutinability and several factors that influence the agglutinability of cells have been identified (Nicolson 1976; Mehta et al. 1988; Pestonjamasp and Mehta 1991). The major factors responsible for agglutination

Correspondence to: Sadashiv M. Gokhale, School of Biochemistry, Devi Ahilya University, Khandwa Road, Indore 452017, India
E-mail: gokhale.drsm@gmail.com

are the number and characteristics of the lectin receptors, the cell-surface charge as determined by sialic acid residues, the mobility of the lectin-receptor complexes in the membrane and the mobility-modulating agents such as cytoskeletal elements, and cellular deformability. A considerable amount of information is available on the concanavalin A (Con A)-mediated agglutination of human erythrocytes after treatment with proteolytic enzymes (Nicolson 1976; Gokhale and Mehta 1987a,b,c), as found in normal nucleated cells (Burger 1970). Presence of sialic acid on glycophorins, especially glycophorin A of erythrocytes appears to be responsible for inhibition of agglutination with Con A (Gokhale and Mehta 1987a). Thus, human erythrocytes offer detailed information of the possible factors which affect agglutination. Band 3 has been identified as Con A receptor in human erythrocytes (Kapito and Lodish 1985). Some factors responsible for Con A-agglutinability of animals like sheep, and rabbit erythrocytes have been reported. Treatments with trypsin and neuraminidase cause the agglutination of rabbit erythrocytes but had almost no effect on sheep erythrocytes (Haskovec and Kinkor 1976). In the present study, we analyzed the actions of proteinases and neuraminidase on erythrocyte surface of two economically important mammals (goat and buffalo) and compared them with that of human and their impact on animal erythrocyte agglutination by Con A.

Materials and Methods

L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin, Tosyl-L-lysine chloromethyl ketone (TLCK)-chymotrypsin, neuraminidase (type VI-from *Clostridium perfringens*), Con A (IV), sodium dodecyl sulfate, phenylmethylsulfonyl fluoride (PMSF), N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylethylenediamine and coomassie brilliant blue R-250 were the products of Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were analytical research grade.

Preparation of erythrocytes

Human blood was obtained from healthy donors of Devi Ahilya University, Indore, India. Goat and buffalo blood of healthy animals was obtained from local slaughter houses. 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 and further washed with 10 volumes of cold Tris buffered saline (TBS; 0.01 M Tris-HCl buffer, pH 7.4 containing 150 mM NaCl) four times at 1000 × *g* value.

Enzyme treatment of erythrocytes

One volume (1 ml) of washed erythrocytes (100% hematocrit/ packed cells) were suspended in two volumes (2 ml) of the enzyme solution (trypsin, chymotrypsin or neuraminidase), gently stirred and incubated at 37°C for 90 min. The trypsin (100 μg/ml) and chymotrypsin (250 μg/ml) were prepared in TBS. Neuraminidase was dissolved in 0.1 M Tris-maleate buffer, pH 5.6 containing 0.11 M NaCl. After incubation, the cells were washed four times with at least ten volumes of chilled TBS (Gokhale and Mehta 1987a).

Membrane preparation

Membranes were prepared from buffer and enzyme-treated erythrocytes according to Hanahan and Ekholm (1974) with the addition of 1 mM PMSF. Washed erythrocytes were lysed by mixing with 30 volumes of cold 0.01 M Tris-HCl buffer, pH 7.4. After 15 min in cold, the suspension was centrifuged at 22,000 × *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 re-centrifuged. In the last washing, 0.05% sodium azide (NaN_3) was added to the washing buffer to prevent microbial growth. In this way, the membranes were washed four times, until a milky white preparation was obtained.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (1970) with some modifications. The slab gel (1.5 mm thickness) consisted of 10% acrylamide in the running gel (pH 8.8) and 5% acrylamide in the stacking gel (pH 6.8). The protein concentration was determined by the procedure of Lowry et al. (1951). Protein samples were solubilized in the sample buffer to get 0.031 M Tris, 1% SDS, 0.25% β-mercaptoethanol and 5% glycerol in final volume and heated at 100°C for 10 min before loading on the gel. Electrophoresis was carried out at a constant current of 2 mA/cm using electrophoresis buffer (0.025 M Tris, 0.2 M glycine, 0.2% SDS).

Staining and destaining of the gels

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 (PAS) staining: The carbohydrate-specific staining was performed according to Fairbanks et al. (1971). 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 room temperature in the following order: 0.5% periodic acid for 2 h; 0.5% sodium arsenite and 5% acetic acid for 30 min; 0.1% sodium arsenite, 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 described 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 Bio-Imaging Systems "LabWorks TM Image Acquisition and Analysis software, version 4.0.0.8".

Agglutination assay

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 room temperature. A control containing the relevant inhibitory sugar (0.05 M α-methyl D-mannopyranoside) and the highest Con A concentration (100 µg/ml), was used in the assay. After 45 min incubation at 37°C, the tubes were tapped several times and the 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 Mehta (1987a) in the following way:

$$\text{Agglutination (\%)} = 100 - \left(100 \times \frac{n_{UC}}{n_{IC}}\right)$$

where n_{UC} is number of unagglutinated cells and n_{IC} is 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 were 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 the statistical evaluation of the results, significance was defined by a probability level of $p < 0.05$. All the experiments were carried out five times.

Results

Proteolytic action of trypsin and chymotrypsin on mammalian erythrocyte membrane proteins

To understand the action of proteinases on surface-exposed membrane proteins, the intact erythrocytes were subjected to proteolytic digestion by treatment either with trypsin or chymotrypsin. The membranes were isolated from extensively washed proteinase-treated erythrocytes and analyzed by SDS-PAGE followed by CBB stain (Fig. 1). The human erythrocyte membrane protein patterns of untreated (Fig. 1, lane 1-H) and trypsin (lane 2-H) treated erythrocytes were almost similar indicating no action of trypsin on erythrocyte surface-exposed proteins. The action of chymotrypsin on band 3 was indicated by the appearance of an intense band of molecular weight (Mr) 58 kDa (Fig. 1, lane 3-H) (Jennings and Nicknish 1985; Gokhale and Mehta 1987a). The densitogram (Fig. 1, DS-H) also indicated the reduction in the peak intensity of band 3 and a sharp increase near the position of band 4.5, Mr 58 kDa. The results of digestion of goat erythrocytes with proteinases indicated a slight action of trypsin possibly on gp155 (Inaba and Maede 1988). Due to the action of trypsin on goat erythrocytes, three new fragments were observed with apparent Mrs of 57 kDa, 48 kDa and 37 kDa (Fig. 1, lane 2-G and DS-G). The high Mr fragments were more intense as compared to lower Mr protein fragments. On the other hand, chymotrypsin had no action on goat erythrocyte membrane proteins. The action of trypsin on buffalo erythrocyte membranes was found to be similar to that of human (Fig. 1, lanes 2-B and 3-B). There was no action of trypsin on buffalo erythrocyte membranes (Fig. 1, lane 2-B) but chymotrypsin showed some action indicated by the appearance of three new degradation products of Mr 63 kDa (greater than band 3 fragment of human - 58 kDa), 50 kDa and 37 kDa fragments (Fig. 1, lane 3-B).

Proteolytic action of trypsin and chymotrypsin on mammalian erythrocyte membrane glycophorins

The actions of proteinases on surface-exposed glycophorins were studied by subjecting the erythrocyte membranes to SDS-PAGE followed by sialoglycoprotein-specific PAS staining (Fig. 2). In case of human erythrocytes, trypsin shows a drastic action on bands PAS-1 and PAS-4 while another band

PAS-2 is partially degraded by the enzyme (Fig. 2, lane 2-H). A new fragment of Mr 62 kDa was seen, possibly indicating the trypsin degradation product of PAS bands, retained in the membrane (Gokhale and Mehta 1987a). In chymotrypsin-treated human erythrocytes (Fig. 2, lane 3-H), a decrease in the intensities of PAS-1 and PAS-2 was observed (Gokhale and Mehta 1987a). In the case of goat erythrocytes, trypsin showed action on PAS-GI, PAS-GII and PAS-GIV bands but it had no action on other PAS bands (Fig. 2, lane 2-G). The degradation products of these glycophorins were observed with apparent Mrs of 150 kDa, 100 kDa, 87 kDa, 65 kDa, 44 kDa and 32 kDa. In case of chymotrypsin-treated erythrocytes (lane 3-G), a drastic action was seen on PAS-GI, PAS-GII, PAS-GIII and PAS-GVIII bands of goat erythrocytes without any action shown on other PAS bands. The degradation products were seen in the range of PAS-GIV to PAS-GVI (99-65 kDa). Densitogram (Fig. 2, DS-G) also showed tremendous decrease in intensities of peaks of PAS-GI and PAS-GII which was an indication of their complete degradation by chymotrypsin. In buffalo erythrocytes, the trypsin acted partially on PAS-BI and PAS-BII but extensively on PAS-BIII, PAS-BIV and PAS-BV (Fig. 2, lane 2-B). Among all the PAS bands of buffalo, PAS-BV was observed as a long diffused zone and these findings were also noticed in the densitogram (Fig. 2, DS-B). Action of chymotrypsin on buffalo erythrocyte glycophorins was indicated by marginal shifting of PAS bands and appearance of some new bands (Fig. 2, lane 3-B).

To know the relative positions of proteins, glycophorins and their degradation products formed due to the action of proteinases on erythrocytes, the PAS-stained gel was restained with CBB stain (Fig. 3). This double stained gel permitted direct visualization of the positions of glycophorin

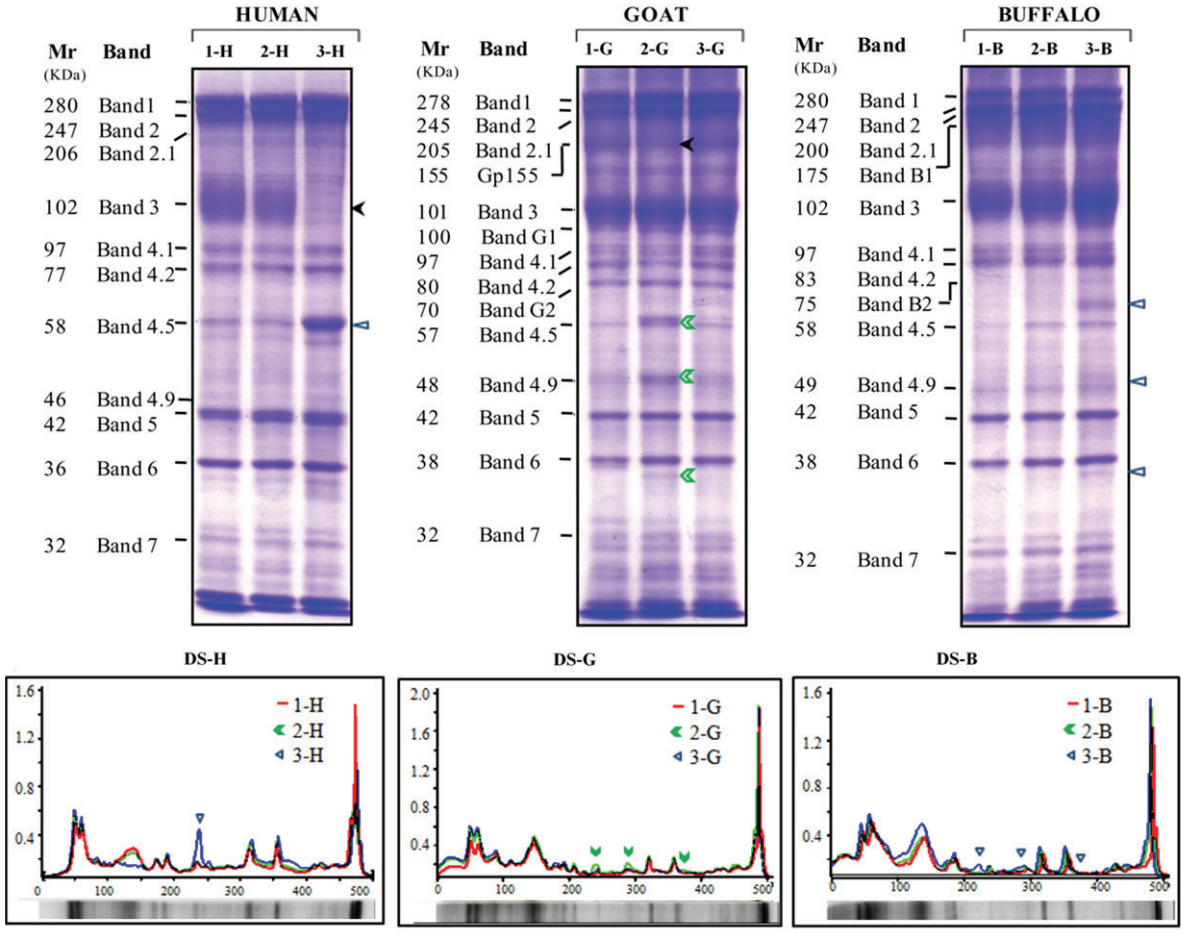

Figure 1. Analysis of membrane proteins obtained from proteinase (trypsin or chymotrypsin)-treated erythrocytes by SDS-PAGE (10% gel) followed by CBB staining. Lane 1: untreated membranes (60 µg protein in each lane); lane 2: trypsinized membranes (60 µg protein in each lane); lane 3: chymotrypsinized membranes (60 µg protein in each lane); DS, densitogram; H, human; G, goat; B, buffalo. (Note: ➤ arrow head indicates the protein acted upon by proteinase; ⋘ and ◅ arrows indicate the new fragments generated after trypsin and chymotrypsin actions, respectively).

bands in relation to all other membrane protein bands. In trypsin-treated (lane 2-H) human erythrocyte membranes (Fig. 3), a new PAS-stained glycophorin fragment (62 kDa) was observed in the region of band 4.5. The new band 3 fragment generated due to chymotrypsin action was found to be present just below the PAS-4 band. The restaining of PAS-stained gel of goat erythrocyte membrane with CBB (Fig. 3) indicated the relative positions of new glycophorin fragments formed by the action of trypsin (lane 2-G) and chymotrypsin (lane 3-G). The membrane glycophorin fragments formed with trypsin treatment were located at various positions viz. below Gp155 (155 kDa), band G1 (100 kDa), band 4.2 (80 kDa), band 4.5 (57 kDa), band 4.9 (48 kDa), above band 6 (38 kDa) and band 7 (32 kDa). The chymotrypsin-treated membrane glycophorin fragments were observed at positions of band G1 (100 kDa), band 4.2 (80 kDa), band G2 (70 kDa) and below band G2 (67 kDa). The trypsin-degraded glycophorin fragments (in restained PAS gel with CBB stain) of buffalo erythrocyte membranes (Fig. 3, lane 2-B) were found to be present below band 6 with Mrs 37 kDa and 34 kDa. The chymotrypsin-degraded glycophorin fragments were located above band 3 (105 kDa), in the region of band 4.9 (49 kDa), below band 5 (41 kDa) and below band 6 with Mr 37 kDa and 34 kDa.

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 can be analyzed by sialoglycoprotein-specific PAS staining. Due to the action of neuraminidase on erythrocyte glycophorins, removal of sialic acid was

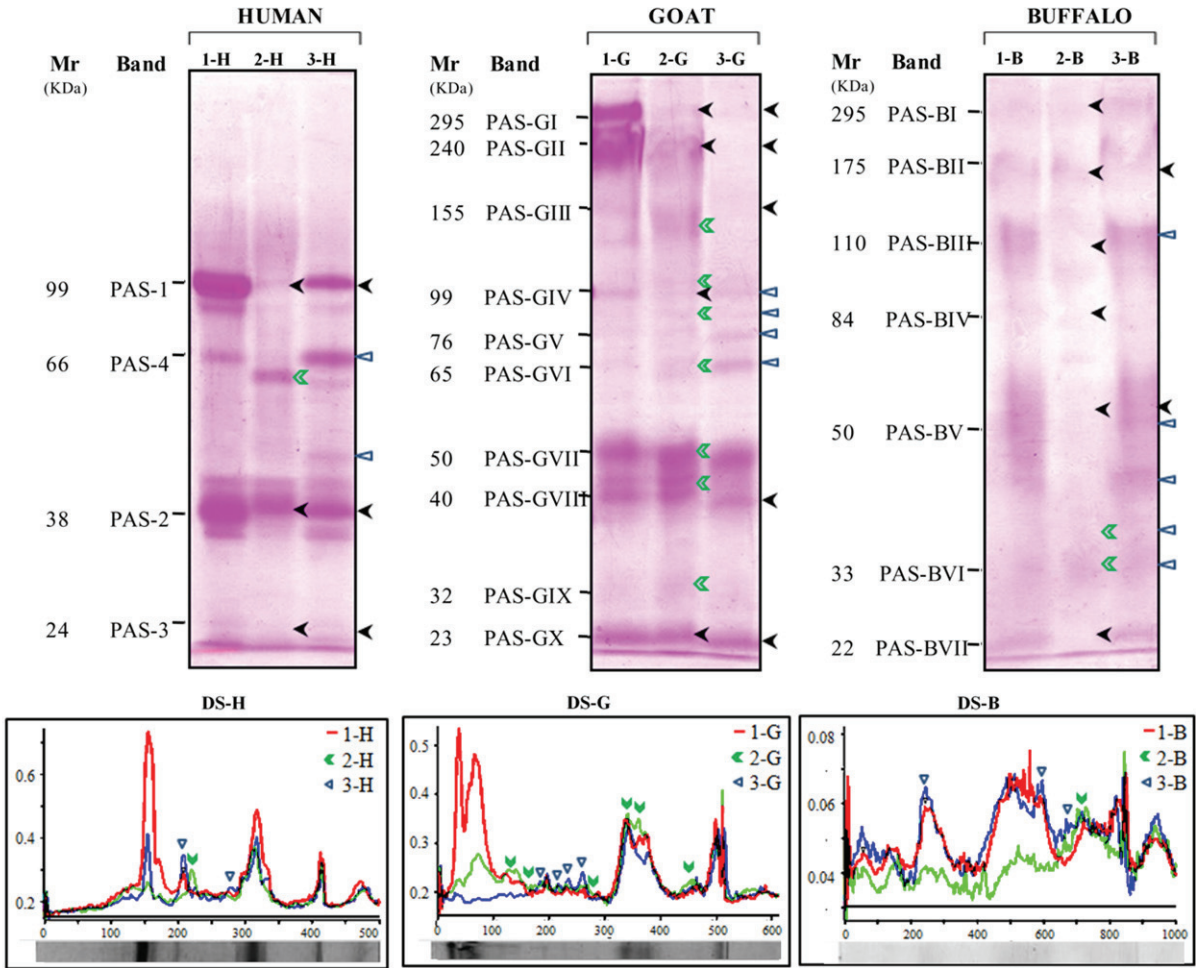

Figure 2. Analysis of membrane glycophorins obtained from proteinase (trypsin or chymotrypsin)-treated erythrocytes by SDS-PAGE (10% gel) followed by PAS staining. Lane 1: untreated membranes (180 µg protein in each lane); lane 2: trypsinized membranes (180 µg protein in each lane); lane 3: chymotrypsinized membranes (180 µg protein in each lane); DS, densitogram; H, human; G, goat; B, buffalo. (see: Note in legend of Figure 1).

indicated by the shift in PAS bands on the polyacrylamide gel (Fig. 4). A remarkable action of neuraminidase on all the PAS bands of human erythrocyte membrane was observed with the appearance of new fuzz bands with Mrs 81 kDa, 35 kDa and 22 kDa (Fig. 4, lane 2-H) as reported earlier (Gokhale and Mehta 1987a). Alteration in the position and intensity of PAS bands is very well depicted in the densitogram (Fig. 4, DS-H). In the case of goat erythrocytes, PAS-GI and PAS-GVII were completely degraded by neuraminidase, as no band was visible at the positions of these glycophorins while PAS-GII did not show any visible change (Fig. 4, lane 2-G). PAS-GIII, PAS-GIX and PAS-GX were partially degraded by neuraminidase as indicated by their reduced intensities. Below PAS-GVII, four new bands (46 kDa, 43 kDa, 38 kDa and 36 kDa) of glycophorins were observed which can be clearly seen in the densitogram (Fig. 4, DS-G). The neuraminidase showed mild action on all the buffalo glycophorins *viz.* PAS-BI to PAS-BVII (Fig. 4, lane 2-B). The surprising observation about buffalo glycophorins was the upward shifts of some PAS bands *viz.* PAS-BIII (120 kDa) and PAS-BV (60 kDa). Other glycophorins like PAS-BI, PAS-BII, PAS-BIV, PAS-BVI and PAS-BVII showed reduced band intensities. These observations regarding upward shifts and decrease in PAS bands' intensities were also seen in the densitogram (Fig. 4, DS-B).

Erythrocyte glycophorins are sialoglycoproteins and due to high sialic acid content they cannot be stained with CBB staining. Alteration in the staining property of glycophorins after removal of sialic acids by neuraminidase was also analyzed by SDS-PAGE followed by CBB staining (Fig. 5). The protein bands in the region of Mrs of 59 kDa and 22 kDa showed higher intensity in neuraminidase-treated human erythrocytes (lane 2-H, DS-H). The neuraminidase action on goat erythrocytes showed the higher intensity of protein bands with Mrs 90 kDa, 50 kDa, 32 kDa and 21 kDa along with an additional new band (75 kDa) (lane 2-G,DS-G). In case of buffalo erythrocytes, neuraminidase digestion showed the appearance of two new protein bands with Mrs 67 kDa and 20 kDa (lane 2-B, DS-B).

Relative positions of erythrocyte membrane proteins and glycophorins after neuraminidase treatment on the same gel were determined by restaining the PAS-stained gel with CBB staining (Fig. 6).

Con A-mediated agglutinability of mammalian erythrocytes

On the erythrocyte membrane surface Con A binds to band 3 (Findlay 1974), but has been found unable to agglutinate native (untreated) human erythrocytes. Only after treatment with a proteolytic enzyme or neuraminidase, the

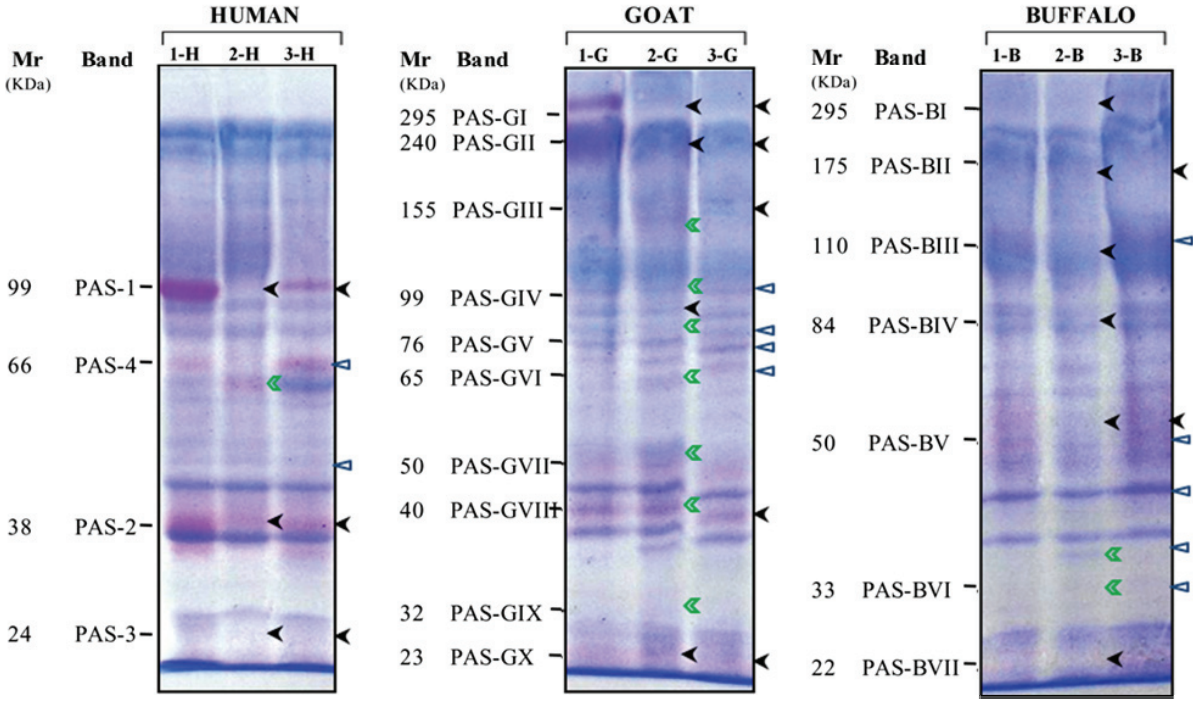

Figure 3. Analysis of membrane proteins and glycoproteins obtained from proteinase (trypsin or chymotrypsin)-treated erythrocytes by SDS-PAGE (10% gel) followed by restaining of PAS-stained gel with CBB staining. Lane 1: untreated membranes (180 μg protein in each lane); lane 2: trypsinized membranes (180 μg protein in each lane); lane 3: chymotrypsinized membranes (180 μg protein in each lane). H, human; G, goat; B, buffalo. (see: Note in legend of Figure 1).

cells become agglutinable with Con A (Gokhale and Mehta 1987a). Con A agglutination behaviour of proteinase and neuraminidase-treated goat and buffalo erythrocytes was studied in comparison to that of human. Con A-mediated agglutinability of mammalian erythrocytes after trypsin, chymotrypsin and neuraminidase treatment was analyzed both under microscope and by microtitre plate assay. The extent of agglutination was determined by counting number of free cells in Con A-treated samples as well as controls. Additional sugar controls were used containing Con A and α-methyl D-mannopyranoside (a sugar derivative), known to inhibit agglutination by binding to Con A with high affinity. Percentage agglutination (Fig. 7) was calculated by substituting these values in the formula as mentioned in Materials and Methods. In microtitre plate assay (Fig. 8), 0C (zero control) in the first row did not contain any 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 as 50 μg/ml and 100 μg/ml, respectively. The microtitre plate assay (Fig. 8) revealed similar results as obtained from microscopic agglutination assay. All mammalian erythrocytes did not show any agglutination in both zero and sugar controls. The mammalian erythrocytes included in this study were able to get agglutinated after trypsin (100 μg/ml), chymotrypsin (250 μg/ml) and neuraminidase (0.01 unit/ml) treatments. A significant variation in the extent of agglutination of erythrocytes was observed among these mammalian species (Fig. 7 and 8).

The extent of agglutination of trypsinized (Tr) erythrocytes was lesser in both goat (GTr) and buffalo (BTr) as compared to human (HTr) (Fig. 7A). The percentage agglutination values for Tr erythrocytes of goat and buffalo with 50 μg/ml Con A concentration were ~17% and 54%, respectively, as

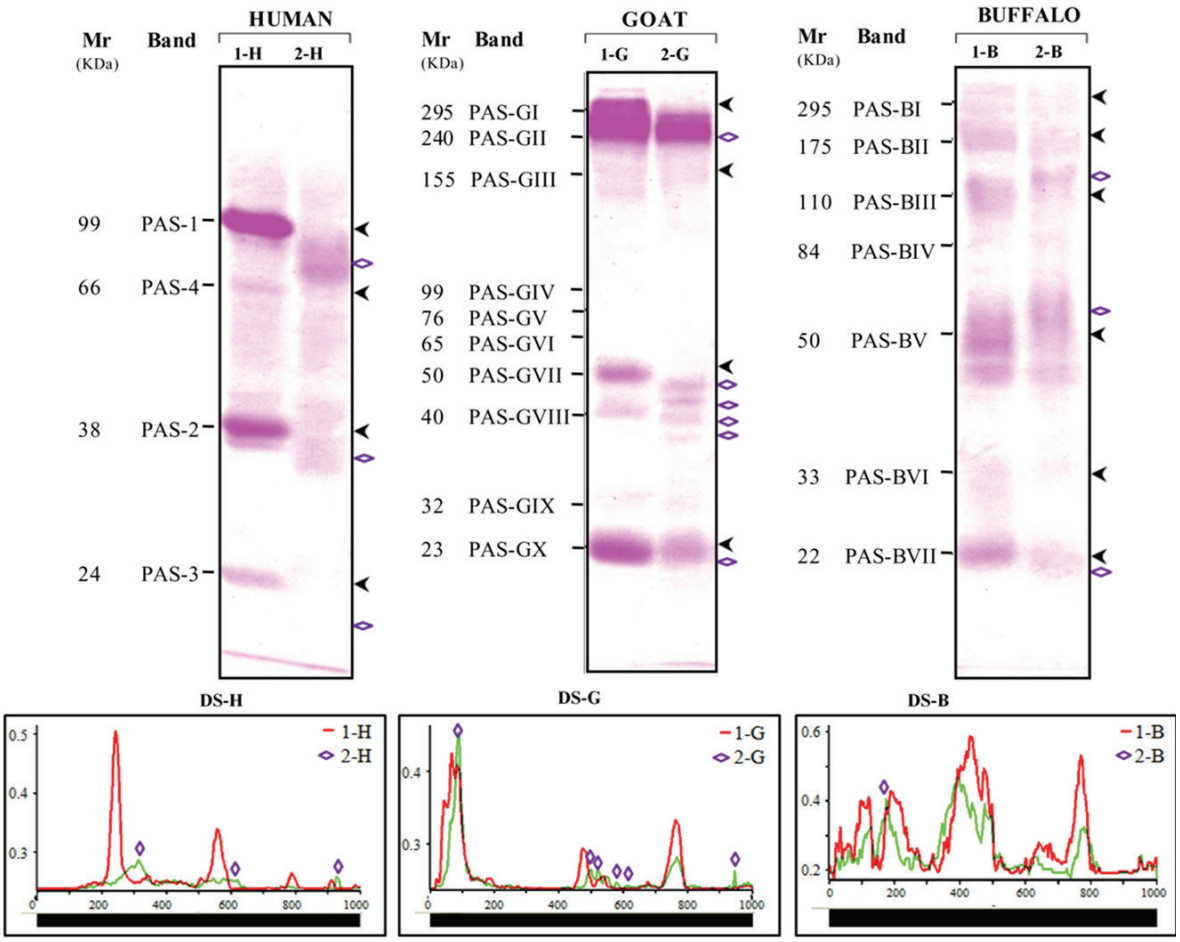

Figure 4. Analysis of membrane glycophorins obtained from neuraminidase-treated erythrocytes by SDS-PAGE (10% gel) followed by PAS staining. Lane 1: untreated membranes (180 μg protein in each lane); lane 2: neuraminidase-treated membranes (180 μg protein in each lane). DS, densitogram; H, human; G, goat; B, buffalo. (Note: ➤ and ◇ arrows indicate the proteins acted upon by neuraminidase and the new fragments generated after its action, respectively).

compared to 62%, obtained with human erythrocytes. These values of percentage agglutination of Tr erythrocytes increased to 45% for goat and 77% for buffalo, as compared to human (85%) when Con A concentration was increased to 100 µg/ml. The agglutination behaviour of all the three mammalian species differed significantly ($p < 0.05$) for Tr erythrocytes. While both HTr and BTr showed higher extent of agglutination, GTr erythrocytes were relatively less agglutinated. The agglutination pattern of trypsin-treated erythrocytes obtained from percentage agglutination data as well as microtitre plate assay was as follows: human > buffalo > goat.

The extent of agglutination of chymotrypsinized (CTr) erythrocytes was higher in goat (GCTr) and buffalo (BCTr) as compared to human (HCTr) (Fig. 7B). The values of percentage agglutination of CTr erythrocytes of goat and buffalo were ~42% and 87%, respectively, as compared to human (39%) with 50 µg/ml Con A concentration and increased to ~63% and 93%, respectively, as compared to human (54%) with 100 µg/ml of Con A concentration. The agglutination behaviour of CTr erythrocytes of all the three mammalian species differed significantly ($p < 0.05$). Whereas BCTr showed higher extent of agglutination, both GCTr and HCTr erythrocytes were less agglutinated. The agglutination pattern of chymotrypsin-treated erythrocytes obtained from percentage agglutination data as well as microtitre plate assay was in the order: buffalo > goat > human.

The extent of agglutination of neuraminidase (Nr)-treated erythrocytes was lesser in both goat (GNr) and buffalo (BNr), being ~43% and 61%, respectively, as compared to human (67%) with 50 µg/ml Con A concentration (Fig. 7C). These values were increased to ~51% and 74%, respectively, as compared to human (85%) with 100 µg/ml Con A concentration. The agglutination behaviour of Nr-treated erythrocytes of all the three mammalian species differed significantly

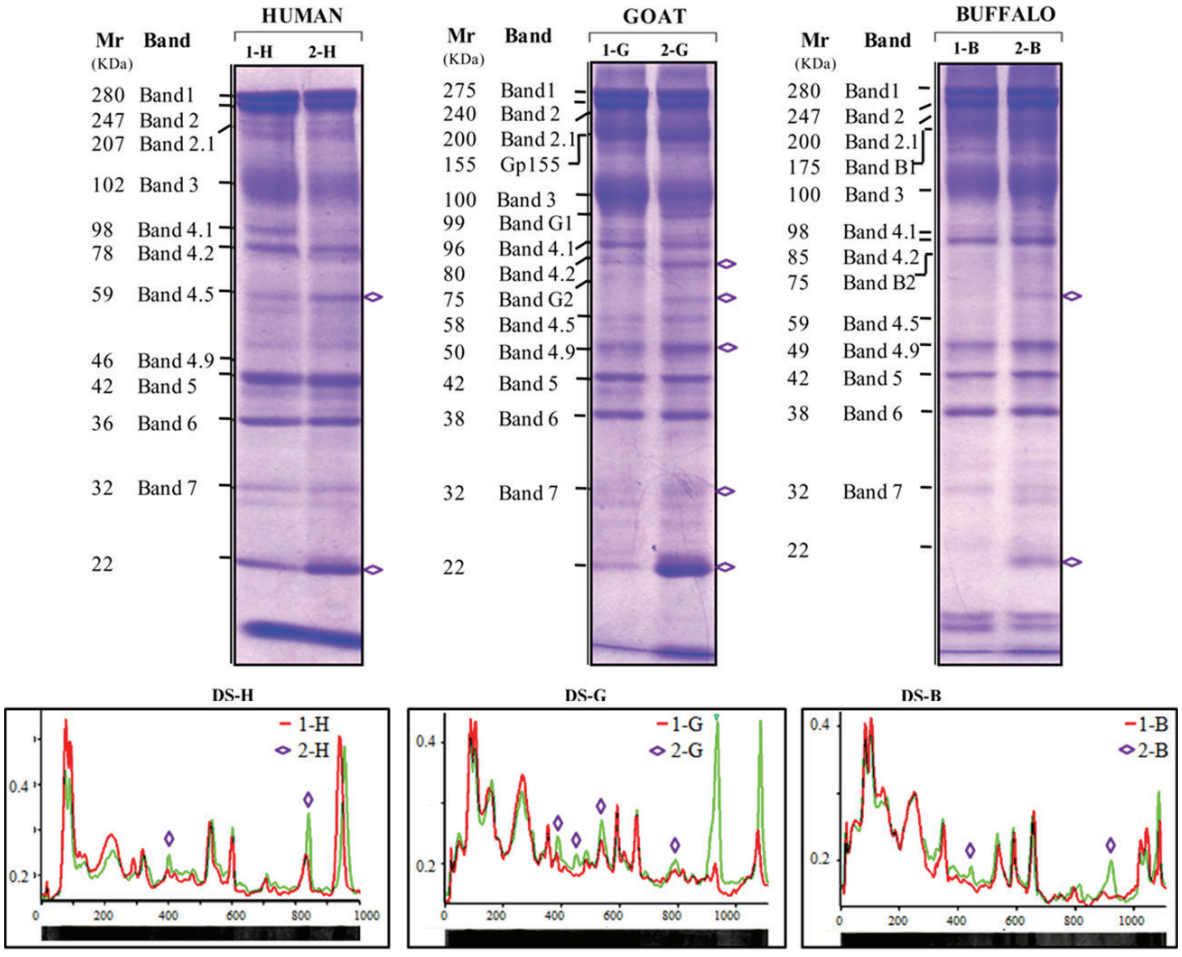

Figure 5. Analysis of membrane proteins obtained from neuraminidase-treated erythrocytes by SDS-PAGE (10% gel) followed by CBB staining. Lane 1: untreated membranes (60 µg protein in each lane); lane 2: neuraminidase-treated membranes (60 µg protein in each lane). DS, densitogram; H, human; G, goat; B, buffalo. (Note: <> arrows indicate the change in intensities of proteins or new fragments generated after the action of neuraminidase).

($p < 0.05$). Both HNr and BNr showed apparently high extent of agglutination, while GNr erythrocytes were relatively less agglutinated with the same concentration of Con A. The agglutination pattern of Nr-treated erythrocytes obtained from percentage agglutination data as well as microtitre plate assay was in the order: human > buffalo > goat.

Discussion

The proteins of human erythrocyte membrane have been extensively studied with respect to their structure and organization in last three decades (Gratzer 1981; Kakhniashvili et al. 2004). Over 250 blood group determinants are known and most of these are located on integral erythrocyte proteins and glycoproteins. The functions of some of these structures are known (Pasini et al. 2010). Glycophorins of human erythrocytes serve as receptors for viruses, bacteria and parasites (Kumar et al. 2006; Spring 2008). Cell surface features have also been studied with respect to the action of proteinases and Nr (Dzandu et al. 1985; Gokhale and Mehta 1987a) followed by lectin agglutinability of human erythrocytes (Nicolson 1976; Gokhale and Mehta 1987a,b,c). There are few reports available related to the structural features of erythrocyte proteins and glycoproteins of non-human mammals (Barker 1991; Matei et al. 2000). In this work attempts have been made to understand the differences in the structural features of surface-exposed membrane proteins and glycophorins of goat and buffalo in comparison to that of human erythrocytes. As reported earlier that glycophorins of erythrocyte membranes of these three species showed marked differences in their number and sizes when visualized by sialoglycoprotein-specific PAS staining (Sharma and Gokhale 2011). Sialic acid residues of glycophorins exposed to exterior face contribute to the cell surface charge of erythrocytes and any variation in the sialic acid content as indicated by PAS-stained gels would be responsible for differences in the cell surface charge among these species.

In this study, action of proteinases *viz.* trypsin and chymotrypsin, on goat and buffalo erythrocytes followed by analysis of their membranes by SDS-PAGE indicated that there was a prominent action of trypsin on goat and chymotrypsin on buffalo erythrocyte membrane proteins (stained with CBB; Fig. 1). There was no action of trypsin on buffalo and chymotrypsin on goat erythrocyte membrane proteins as indicated by CBB-stained gel. GTr erythrocyte membranes showed three new fragments, one of these was identified as 57 kDa fragment of gp155 glycoprotein (Inaba and Maede

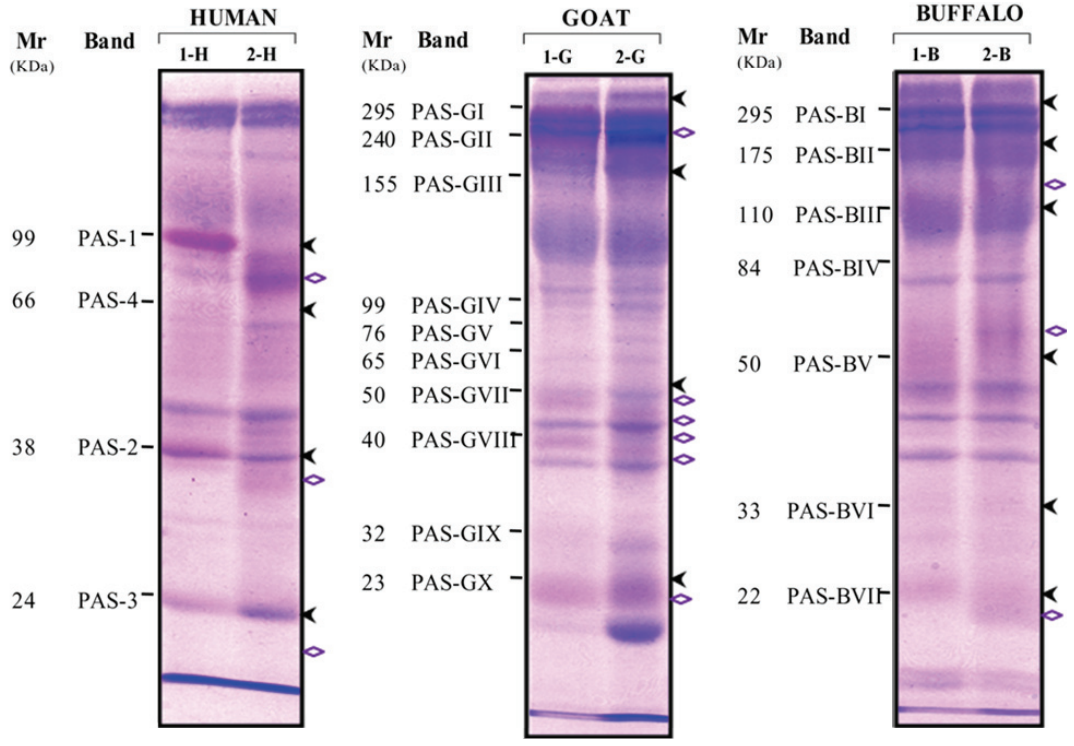

Figure 6. Analysis of membrane proteins and glycophorins obtained from neuraminidase-treated erythrocytes by SDS-PAGE (10% gel) followed by restaining of PAS-stained gel with CBB staining. Lane 1: untreated membranes (180 µg protein in each lane); lane 2: neuraminidase-treated membranes (180 µg protein in each lane). H, human; G, goat; B, buffalo. (see: Note in legend of Figure 4).

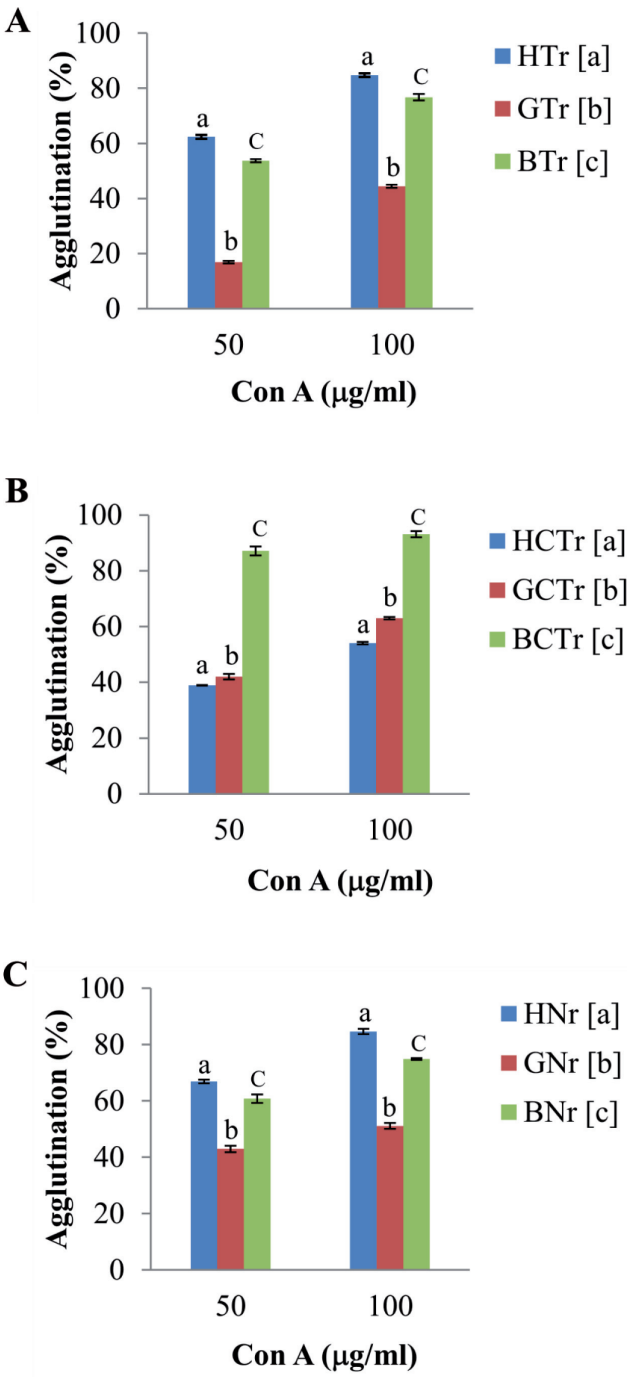

Figure 7. Con A-mediated agglutinability of human (H), goat (G) and buffalo (B) erythrocytes treated with different enzymes (determined by microscopic agglutination assay). Graphs A, B and C are showing percent agglutination of trypsinized (Tr), chymotrypsinized (CTr) and neuraminidase (Nr)-treated erythrocytes, respectively. Con A concentration used were 50 and 100 µg/ml. The data are mean of ± S.E.M. ($p < 0.05$; $n = 5$). The upper symbols with small letters indicate the differences in Con A agglutinability among three mammalian species at Con A concentration of 50 and 100 µg/ml, respectively.

1988). This probably indicated that there were some lysine and/or arginine residues of proteins exposed on cell surface for trypsin action in goat which were not available in case of human and buffalo erythrocytes. Similarly, chymotrypsin showed action on aromatic/hydrophobic amino acid residues of human and buffalo erythrocytes but not on goat erythrocyte surface proteins. There are reports about the action of chymotrypsin on band 3 of human erythrocytes exposed to cell surface (Markowitz and Marchesi 1981). BCTr erythrocyte surface proteins showed three new fragments of Mr 63 kDa, 50 kDa and 37 kDa. Action of proteinases on glycophorins of human erythrocyte membranes were in agreement with previous literature (Dzandu et al. 1985; Gokhale and Mehta 1987a). Trypsin showed action on many glycophorins of human and buffalo erythrocytes. On the other hand, chymotrypsin did show partial or no action on these glycophorins. In the case of goat erythrocytes, trypsin and chymotrypsin both showed the action on many glycophorins i.e. PAS GI to GIV. The only difference in the action of these two enzymes was that trypsin showed a partial while chymotrypsin showed a drastic action on goat erythrocyte glycophorins. The Nr-treated goat and buffalo 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 the PAS banding pattern confirmed the action of Nr on sialic acid residues of glycophorins exposed to cell surface, similar to human erythrocytes (Gahmberg and Andersson 1982).

The mammalian erythrocytes became agglutinable with Con A after treatment with proteinases or neuraminidase. The actions of the enzymes on band 3 did not correlate with their abilities to increase the Con A agglutinability as chymotrypsin-treated human erythrocytes (showing marked action on band 3) showed minimum agglutination among these three species. On the other hand, CTr buffalo erythrocytes (with no action on band 3) showed maximum agglutination with Con A. It is possible that the ability of Con A to promote agglutination was related to the extent of degradation of glycophorins, specifically PAS bands GI and GII of goat, and BI, BIII and BV of buffalo erythrocytes. As indicated by PAS-stained gels, a direct correlation was found between degradation of glycophorins and Con A agglutinability similar to human erythrocytes (Gahmberg and Andersson 1982; Gokhale and Mehta 1987a). Action of Nr specifically demonstrated the negative role of cell surface sialic acids on Con A agglutinability, similar to human erythrocytes. Since glycophorins carry a very large proportion of the total sialic acids of erythrocyte membrane, their removal would decrease the overall surface charge and thus facilitate the approach and interaction between the cells during agglutination. In fact, this is also a requirement since for stable interaction; the cells have to come within 0.5 nm in the vicinity of each other, which is initially impossible because of the potential

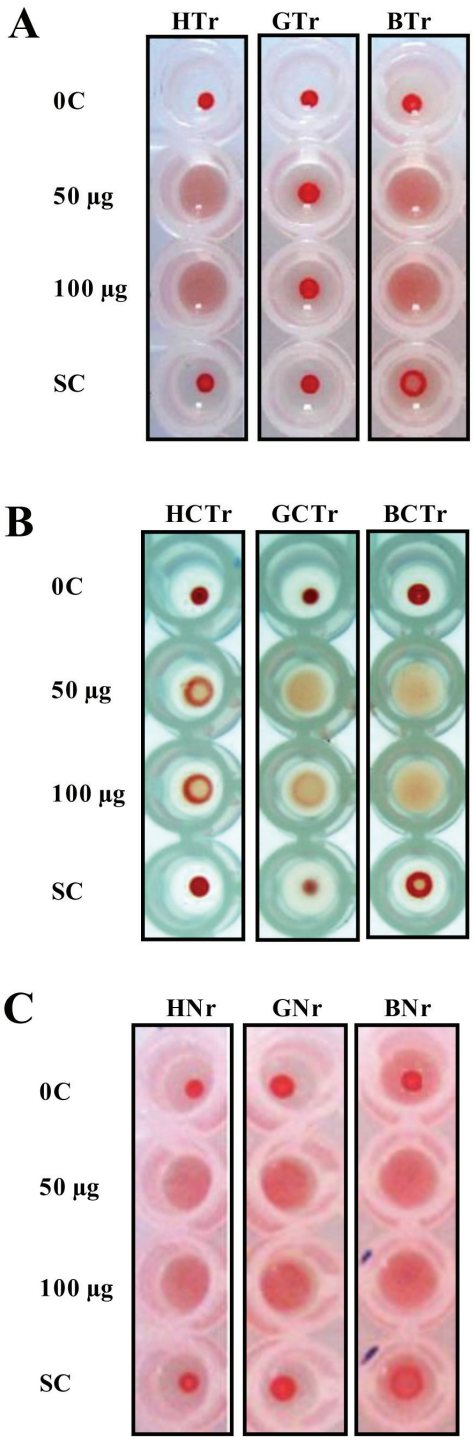

Figure 8. Microtitre plate assay of Con A-mediated agglutinability of human (H), goat (G) and buffalo (B) erythrocytes treated with different enzymes. Figures A, B and C show agglutination assay of trypsinized (Tr), chymotrypsinized (CTr) and neuraminidase (Nr)-treated erythrocytes respectively. Con A concentration used were 50 and 100 µg/ml. 0C (zero control without Con A), SC (sugar control with 100 µg/ml Con A and 0.05 M α-methyl D-mannopyranoside).

energy repulsion barriers. In order to overcome this, sensing of surface regions having lower than average charge density would be needed (Weiss 1973). As Con A is known to have maximum affinity for binding to mannose and glucose (Goldstein and Hughes 1978), these sugars determine the number of Con A binding sites on the erythrocyte cell surface. Therefore, another possible reason for enhancement of agglutination may be the exposure of such Con A binding sites, which were hidden prior to the enzyme treatments. Present study is important for understanding the structural features of goat and buffalo erythrocyte membrane proteins especially with respect to differences in the characteristics as compared to human erythrocytes. It will be helpful in investigating the surface architecture of glycophorins of goat and buffalo erythrocyte membranes.

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