ABO reverse grouping: effect of varying concentrations of the enzyme bromelain

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ABSTRACT

Proteolytic enzymes can be extremely useful for the identification of clinically significant antibodies in blood grouping; however, they can also be destructive to certain agglutination reactions. ABO antibodies are the most clinically significant. A review of the literature reveals little research involving proteolytic enzymes and IgM antibodies. This study investigates the effects of various concentrations of the proteolytic enzyme bromelain on the agglutination of reagent A_1 and B cells with donor plasma during ABO reverse grouping on the Olympus PK 7300 automated serology analyser. The optimum bromelain concentration improved or enhanced antigen-antibody reactions, causing a reduction in ABO failures. The results were analysed using an ANOVA test. An anomalous result was obtained at 0% bromelain where less ABO failures were generated than expected. Although most results were not statistically significant, the optimum bromelain concentration for ABO reverse grouping indicated was 0.25%. This work also highlighted the fact that ABO failures can be attributed to other factors such as sensitivity of reagent cells and antibody avidity. This study identified limitations and problems with the methods used and presents recommendations for future research which may assist in the clarification of the role of proteolytic enzymes in ABO reverse grouping.

KEY WORDS: Blood grouping and crossmatching.
Bromelains.
Peptide hydrolases.

Introduction

Proteolytic enzymes lyse proteins by breaking peptide bonds. They are used widely in red cell serology as they cause modifications to the red cell membrane. Proteolytic enzymes can be used to pretreat reagent red cells or as a diluent medium for reagents and blood samples.

Enzyme methods are used to destroy certain antigens to aid the agglutination and identification of otherwise non-agglutinating antibodies.[1–9] However, the removal of some of the red blood cell (RBC) membrane material is non-specific and so some antigens are completely cleaved from the membrane surface, reducing or completely preventing potential antigen-antibody reactions; thus, enzyme treatment can be detrimental to results.[10] Occasionally, however, antigens are only damaged when a sufficiently high concentration of enzyme is used.[11]

Proteolytic enzymes used commonly in red cell serology include bromelain, papain and ficin.[6,8,9,12–14] Each may be selected for its availability, cost and for its specific mode and molecular site of action. Enzyme methods are quick and simple to perform but the results obtained tend to vary widely between laboratories.[1,10] The optimum bromelain concentration for use in red cell serology is suggested to lie between 0.2% and 0.5%.[15]

Most red cell antigens are glycoproteins, which are long-chain structures that extend above the RBC membrane surface.[16] One such glycoprotein, sialoglycoprotein, contains most of the red cell sialic acid (e.g., N-acetyl-neuraminic acid) and is one of the major charged molecules of the RBC membrane, conferring a net negative charge (the cell surface potential) on the red cell.[1,6,17] It is this net negative charge that can prevent certain red cell antigens from agglutinating in a saline medium.

Proteolytic enzymes break the peptide bonds of glycoprotein molecules in the RBC membrane when used at a sufficiently high concentration.[7,18] This removes varying amounts of protein and sialic acid from the red cell surface and reduces the net negative charge of each red cell and reduces steric hindrance.[1,8] Thus, each red cell attracts fewer ions from the suspending medium (usually saline) and reduces the zeta potential.[19] The minimum distance between red cells decreases, permitting them to move closer together.[6,8] This allows some antigens to become more accessible to their corresponding antibody, thereby allowing these cells to agglutinate in a saline medium.

In the Donor Testing Laboratory at Brentwood, the majority of red cell serology is performed on an Olympus PK 7300 fully automated serology analyser, and bromelain is used as a diluent to enhance red cell serological reactions and reduce the number of sample failures.

Proteolytic enzymes are useful for the identification of clinically significant antibodies, with ABO antibodies being the most clinically significant in transfusion. Thus, the rationale for this study is to investigate the effect of various concentrations of bromelain on the agglutination of reagent A_1 cells and B cells with donor plasma of differing ABO groups in order to determine whether or not the reverse ABO grouping reactions can be improved. The study also aims to determine the optimum concentration of bromelain for the reverse ABO reaction to occur on the Olympus PK 7300 analyser, as different machines tend to have different

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optimal working conditions and the literature implies that these enzymes are used specifically for the identification of IgG Rh antibodies.[6,11] It also attempts to determine whether or not failures are due to a false reaction type (e.g., false positive, false negative or query) or the reagent cell channel (e.g., A_1 or B).

The study also tests the hypothesis that increasing the concentration of bromelain will enhance the antigen-antibody reaction between reagent A_1 and B cells and donor plasma, up to an optimum concentration, after which any further increase in concentration will be detrimental to the reaction.

The four measurements taken on the Olympus PK 7300 are SPC, P/C, LIA and BG/C. The main parameters used for statistical analysis are SPC, which measures the 'sharpness between the peripheral and central regions' (i.e., the measurement at the border of the negative cell button, between the bright and dark region) and LIA (low intensity area), a measure of the area of cells at the centre of the well. P/C is the ratio of transmitted light at the peripheral region to the central region (e.g., P/C = [P/C] x 10). BG/C is a background measurement and is the ratio of transmitted light outside to that through the centre of the well, and is normally used to detect empty wells. SPC, LIA and the overall number of ABO failures per bromelain concentration were used to interpret the results.

Materials and methods

This study used donor plasma samples in a prospective single-centre randomised, controlled trial of the effect of varying concentrations of bromelain on reverse ABO grouping using the Olympus PK 7300 automated serology analyser. As the samples used were donor plasma samples, they had already undergone ABO and Rh automated serology typing. In this way, problem samples were eliminated and systematic error was reduced.

Table 1. Preparation of bromelain concentrations.

Bromelain concentration (%)	Dilution factor (5% bromelain:PBSS)
0	–
0.0625	1:80
0.125	1:40
0.250	1:20
0.500	1:10

The Olympus PK 7300 was operated following the manufacturer's guidelines for ABO reverse grouping using reagent A_1 and B cells. A total of 500 centrifuged donor plasma samples (supplied by blood donors) and 30 control samples (supplied by known donors) were tested at each bromelain concentration.

Diluents were prepared using 5% bromelain solution supplied by NHSBT Reagents as frozen 25 mL vials. Phosphate-buffered saline solution (PBSS) was supplied by Inverclyde Biologicals. Bromelain concentrations were prepared using the dilutions shown in Table 1.

The 0% bromelain group was used as a control group. The control group used randomly selected donor plasma samples. The selection of samples was randomised to reduce bias.

Once the samples, reagents and diluents were dispensed

Table 2. Failure rates obtained at varying bromelain concentrations.

Bromelain concentration (%)	ABO failures due to:						Total ABO failures	Failure rate (%)
	Reagent A cell reaction			Reagent B cell reaction				
	False +ve	False –ve	?	False+ve	False –ve	?		
0	0	0	1	0	4	9	14	2.8
0.0625	0	0	0	0	10	14	24	4.8
0.125	0	0	4	0	8	21	32*	6.4
0.250	0	1	2	0	4	10	16†	3.2
0.500	2	0	2	0	5	8	17	3.4

*One sample produced equivocal results with both the A and B cell channel.
†One sample was a false negative for both reagent A and B cells.

Table 3. Mean SPC values obtained at varying bromelain concentrations.

Bromelain concentration (%)	Reagent A cell reaction			Reagent B cell reaction		
	False +ve	False –ve	?	False+ve	False –ve	?
0	–	–	15	–	24	12.78
0.0625	–	–	–	–	21.4	12.57
0.125	–	–	13.25	–	22.13	11.05
0.250	–	19	10.5	–	21.5	11.3
0.500	2	–	10	–	18.6	12

The '–' symbol indicates that no reading was obtained in that category.

Table 4. Mean LIA values obtained at varying bromelain concentrations.

Bromelain concentration (%)	Reagent A cell reaction			Reagent B cell reaction		
	False +ve	False –ve	?	False+ve	False –ve	?
0	–	–	207	–	372.75	154.44
0.0625	–	–	–	–	377.1	173.14
0.125	–	–	163.5	–	501.25	164.05
0.250	–	376	154.5	–	456.25	133.9
0.500	0	–	127.5	–	430.6	180.57

The '–' symbol indicates that no reading was obtained in that category.

in the microplate, it was incubated at 30°C for 60 min. The microplates were read and interpreted automatically by the analyser and the results were printed.

Reverse ABO grouping failures are defined as samples that fail to group as a result of errors in the A and B cell channels, channels 11 and 12, respectively. Samples that failed for any other reason were not included in the total number of ABO failures, and thus were not included in the statistical analysis.

Donor consent and confidentiality

Donor confidentiality is protected by the Data Protection Act. Ethical permission was not required for this study as donors give consent during their donation to allow their samples to be used for research purposes where required.

Statistical analysis

The SPC and LIA values for false-positive, false-negative and query reactions at each bromelain concentration are presented as mean values. Variation was determined by an analysis of variance (ANOVA) statistical test. Statistical significance was set at $P \leq 0.05$.

Results

A summary of the main findings are shown in Tables 2, 3 and 4. The total number of ABO failures at 0% bromelain was lower than expected and generated the lowest failure rate in this study. Generally, as the concentration of bromelain increased, reagent B cell reactions improved (fewer false-negative reactions). The reagent A cell reactions were not affected by bromelain concentration until it reached 0.5%, which resulted in two false-positive reactions.

Results for the number of repeats are shown in Table 2. There was a statistically significant relationship between the number of ABO failures generated due to reagent A cells and reagent B cells, and also a statistically significant relationship between the reaction types (e.g., false positive, false negative, query) that caused ABO failures. However, no statistically significant relationship was found between the concentration of bromelain and the number of ABO failures. However, there was a statistically significant variation between all data ($P<0.05$).

There was a statistically significant relationship between the SPC values for failed samples and the reagent cell (A or B) channel, and a statistically significant relationship between SPC values generated by the reaction types that caused ABO failures. There was no statistically significant difference between the SPC values for failed samples and the bromelain concentration. Overall, for mean SPC values of failed samples, there was a statistically significant variation between all data ($P<0.05$).

Average LIA values are shown in Table 4. There was no statistically significant relationship between LIA values for failed samples and the reagent cell (A or B) channel, but there was a statistically significant relationship between LIA values generated by the false-reaction types that caused ABO failures. There was no statistically significant difference between LIA values for failed samples and bromelain concentration. Overall, for the mean LIA values of failed samples, there was a statistically significant variation between all data ($P<0.05$).

Unusual values generated for the ANOVA tests performed using mean LIA values for failures of different reaction types, in either the reagent A cell channel or the reagent B cell channel, were due to the presence of blank cells (i.e., some categories did not generate failures) in the Excel spreadsheet. A zero could not be entered in these cells as they were classed as genuine LIA readings generated by the analyser. However, zero could be entered in empty Excel cells for SPC values, as the reading is the ratio of the number of light pixels moving through the plate between the peripheral and central regions, and readings of zero cannot be obtained.

Discussion

Investigating the effect of varying the concentration of bromelain on ABO reverse grouping provided some interesting results. A significant relationship was seen between the number of ABO failures generated and the reagent cell type, and also between the number of ABO failures and the reaction type (e.g., false positive, false negative or query). However, there was no statistically significant relationship between the number of ABO failures generated and the bromelain concentration. However, other research indicates that there is a relationship between the ABO antigen-antibody reaction and the concentration of enzyme (bromelain) used.[20,21]

As there are a greater number of antigen sites on A_1 cells ($\sim 0.81–1.17 \times 10^6$) than on B cells ($0.75 \times 10^6$), reagent B cells tend to generate more ABO failures during ABO reverse grouping.[11] From the failure rates obtained at varying bromelain concentrations (Table 2), as concentration increases generally reagent B cell reactions are improved (fewer false negatives in this category).

The results obtained for the 0% bromelain group were better than expected as less ABO failures were generated. This anomaly may be due to the fact that this run was performed using a particularly sensitive batch of reagent B cells. Sensitivity is defined by the number of antigen sites on the reagent cell, thus more-sensitive batches have a greater number of antigen sites and therefore induce stronger antigen-antibody reactions. This study highlights the fact that the reagent cell sensitivity may play an equivalent role to bromelain concentration in successful ABO reverse grouping. The density of B epitopes on the cell surface and the structure of the various glycoconjugates to which they are attached affects the ability of anti-B antibodies to interact with normal B and B subgroup red cells.[20]

At 0.5% bromelain, the reagent A cells appeared to be over-sensitised by the enzyme as there were two false-positive reactions at this concentration. Although antibody detection increases with increased bromelain concentration, false-positive reactions are also increased.[13,14] At this concentration, the negative charge on the red cell is decreased such that the cells are able to move close together and 'clump' without the aid of an antigen-antibody reaction. This shows that 0.5% is above the optimum bromelain concentration for ABO reverse grouping.

From the results generated in this study, it appears that 0.25% bromelain is the optimum concentration for reverse ABO grouping on the Olympus PK 7300. Another study indicates that the optimum bromelain concentration for use in red cell serology lies between 0.2% and 0.5%,[15] which supports the results presented here.

Comparing the results obtained with 0.125% bromelain (the concentration in current routine use) with those obtained using 0.25% bromelain, it can be seen that the number of ABO failures generated are approximately double those found at 0.25% (i.e., approximately twice as many reagent A cell channel queries and reagent B cell channel false negatives and queries). Overall, the number of ABO failures generated with 0.125% bromelain was 32 (6.4%) compared to 16 failures (3.2%) with the 0.25% concentration.

The number and distribution of red cell antigens on the red cell membrane vary between individuals and this affects the strength of the agglutination reaction.[20] In the same way, the avidity of anti-A, anti-B and anti-A,B in the immune system varies between individuals, thus affecting reagent red cell agglutination and making some ABO failures inevitable.

Red cells have multiple repeating antigenic sites and their respective antibodies contain multiple binding sites. The interaction between antigen and antibody at one site will increase the probability of an antigen-antibody reaction at another site. The strength of the interaction between multivalent antibody and antigen is known as avidity.[22]

Generally, the use of bromelain in ABO grouping increases avidity and thus improves the antigen-antibody reaction.[23] However, some low-avidity samples may fail in reverse ABO grouping at all bromelain concentrations used and thus may have influenced the results obtained in this study. Therefore, conducting all experiments on the same batch of donor samples may prove beneficial to future research of this kind, as these problematic low-avidity samples could be accounted for and eliminated from statistical analysis. Ascertaining the optimum bromelain concentration for ABO reverse grouping may assist in optimising the antigen-antibody reaction in samples with low ABO antibody avidity and thus assist in the generation of valid results.

Although the use of proteolytic enzymes in red cell serology is widely documented, most literature implies that these enzymes are used specifically to enhance the identification of IgG antibodies, particularly Rh antibodies, and little is mentioned about the use of proteolytic enzymes for the identification of naturally-occurring ABO antibodies.

Generally, IgM ABO antibodies have a relatively high avidity compared to IgG antibodies,[22] which is perhaps why little research has been undertaken into the use of proteolytic enzymes in ABO grouping. Rh D typically has about 30,000 antigen binding sites per red cell, which is considerably less than the number of antigen binding sites for ABO antigens.[24] However, one study used the proteolytic enzyme ficin (1%) successfully to show the presence of a weak variant of B antigen (B_w) on the erythrocytes of a child. This proved that a weak variant of B existed on either the mother's or the father's erythrocytes (originally thought to be blood group A and blood group O, respectively) and thus confirmed parenthood.[20] Another study showed that bromelain has a significant effect on ABO reverse-grouping reactions when a microplate agglutination method was devised for blood grouping and reverse typing without the need for centrifugation.[21]

From the mean SPC values obtained at varying bromelain concentrations (Table 3), it can be seen that the reaction between reagent cells and donor plasma is improved and cell agglutination is improved as bromelain concentration increases. This is shown by the general decrease in the mean SPC values for false negatives and queries towards the positive threshold range (1–8) as bromelain concentration increases

From the mean LIA values obtained at varying bromelain concentrations (Table 4), it can be seen that there is a decrease in the mean LIA values for the reagent A cell channel queries as bromelain concentration increases.

As the number of ABO repeats and their mean SPC and LIA values at varying bromelain concentration are not statistically significant, perhaps 500 samples at each bromelain concentration was insufficient to show statistical significance. Although the results obtained in this study may not be statistically significant, they are important from a laboratory point of view, as 500 samples is the maximum number that can be tested in one continuous batch.

Further clarification of the effect of varying the bromelain concentration in ABO reverse grouping could be obtained by using reagent cells of a single batch at all concentrations tested. In this way, biased results due to cell sensitivity would be eliminated. It would also ensure that the reagent cells are of the same age, which is important because the membrane becomes more fragile as the cells age. As bromelain causes damage to the red cell membrane, its effect on aged RBCs could result in an increased number of ABO failures.

This study only examined plasma samples on the Olympus PK 7300 automated serology analyser. Therefore, further research is required to discover whether the importance of bromelain concentration is more specific to a particular analyser or to the ABO antigen-antibody reaction.

It was hypothesised that the antigen-antibody reaction between reagent A_1 and B cells and donor plasma would be enhanced by increasing the concentration of the proteolytic

enzyme bromelain, up to an optimum concentration, after which further increases would be detrimental to the reaction. Although this study did find a statistically significant relationship between bromelain concentration and the number of ABO failures, other available evidence suggests that the optimum bromelain concentration for reverse ABO grouping is 0.25%. However, statistically significant relationships were apparent between the number of ABO failures generated and the reagent cell type (A_1 or B) and the reaction types (i.e., false positive, false negative, query) that cause ABO failures. This study did, however, highlight errors in the methods used and thus further studies are required to clarify the relationship between variables. □

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