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CRYOPRESERVATION OF SHEEP RED BLOOD CELLS

2. PURIFIED POLYVINYLPYRROLIDONE AND HYDROLYZED STARCH AS PROTECTIVE AGENTS

By

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MYHRVOLD, VESLEMØY: *Cryopreservation of sheep red blood cells. 2. Purified polyvinylpyrrolidone and hydrolyzed starch as protective agents*. Acta vet. scand. 1979, *20*, 531—536. — The protection of sheep erythrocytes against damage at low temperature (—196°C) was investigated using purified polyvinylpyrrolidone (PVP) and hydrolyzed starch as cryoprotective agents. Identical results were obtained with untreated PVP, neutralized PVP and PVP purified by chromatography. With hydrolyzed starch the cryoprotection was dependent on the type and concentration of the starch used and on the extent of hydrolysis of the starch prior to use. Cell recovery with some starch types was the same (80—85 %) as that obtained with PVP.

sheep red cells; preservation; low temperature.

Sheep red blood cells are useful as reagent for assays in serology and immunopathology. The normal functional life of such cells prepared for titrations of complement components is only about one week when stored at 4°C (*Myhrvold* 1979 b) requiring weekly preparations. This is time-consuming and also leads to many cells being wasted if they are not used within this time interval. Any extension of the functional life of sheep red blood cells is therefore useful.

Earlier experiments (*Myhrvold* 1979 a) showed that preparations of polyvinylpyrrolidone (PVP), with an average molecular weight of 10 000, were well suited as cryoprotectant for storage of sheep red cells in liquid nitrogen. A cell recovery of

80—85 % was achieved. Unbuffered 10 % solution of the commercial PVP had a pH of 4.2—4.6. For cryoprotection of a strain of Pseudomonas better results were obtained using purified PVP preparations from which a low molecular acidic substance had been removed by column chromatography (*Ashwood-Smith & Warby* 1971). The present paper is concerned with cryoprotection of sheep red cells with untreated PVP, neutralized PVP and PVP purified by different chromatographic methods. In addition the cryoprotective effect of starch products has been investigated. Hydroxyethyl starch (HES) protects human red cells well and is completely metabolized in the human body (*Robson* 1970).

MATERIALS AND METHODS

Purification of PVP

Four ml of a 40 % (w/v) solution of PVP K 15 (average molecular weight 10 000, Fluka AG) in water was chromatographed on columns (1.4 × 40 cm) of Sephadex G 25, Sephadex G 50, and AB 11A8 Ion Retardation Resin (Bio-Rad Laboratories) with water as eluant. Three ml fractions were collected, and, after dilution 1:1000, OD was read at 205 nm for estimation of PVP. Fractions containing PVP were processed to dryness on a Büchi Rotavapor "R" No. 16192 and dissolved in 0.15 M-NaCl to a 20 % solution.

The cryoprotective effect of chromatographed PVP was compared with PVP adjusted to pH 7.3 with 4 M-NaOH and with untreated PVP.

Hydrolyzed starch

The starting material included four preparations: Avelex 1030, Avelex 2530, Perfectamyl A 25/A 75 and Amylogum CL-S (Avebe G.A. Holland). Avelex 1030 and Avelex 2530 are potato starches carboxylated with hypochlorite and etherized by treatment with ethylen oxide. The viscosity is high for Avelex 1030, middle to low for Avelex 2530. Perfectamyl A 25/A 75 is a hydroxypropylated potato starch, and Amylogum CL-S is a gum product with low viscosity.

Avelex 1030, Avelex 2530 and Perfectamyl A 25/A 75 were not soluble in desired concentrations and were therefore hydrolyzed before use as follows. One hundred ml 40 % starch in 0.15 M-HCl were heated for varying times in a 100°C water bath in a 100 ml

flask. After hydrolysis the products were neutralized with 4 M-NaOH, diluted to desired concentrations in 0.15 M-NaCl and the viscosity determined by the Ostwald procedure. The cryoprotective effect of the products was investigated. Amylogum CL-S was prepared as a 28 % stock solution and when desired diluted in 0.15 M-NaCl.

Sheep red blood cells

Sheep red cells collected in acid-citrate-dextrose (ACD) (*Myhrvold* 1979 a) were washed two to five times with eight volumes of veronal-buffered-sucrose ($GVBSM^{++}$-sucrose) prepared as follows: 486.1 g sucrose, 5.095 g Na-5,5 diethyl barbiturate and 18 g NaCl were dissolved in about 1.5 l of distilled water. pH was adjusted to 7.35 ± 0.05 with 1 M-HCl, the acid being added slowly with vigorous mixing of the solution. Five g gelatin dissolved in distilled water and 2.5 ml of a solution containing 1.0 M-$MgCl_2$ and 0.15 M-$CaCl_2$ were added. The volume was brought to 2 l with distilled water. This stock solution was kept at —20°C and diluted two and a half times with distilled water before use. The cells were suspended to 4×10^9 cells/ml in $GVBSM^{++}$-sucrose, and to one volume of the suspended cells was added one volume cryoprotectant, the mixture distributed (1.5 ml) into 2 ml plastic serum test tubes No. 1076 (Nunc) and plunged directly into liquid nitrogen. Thawing was performed in a 43°C water bath and the loss of cells calculated from degree of hemolysis (*Myhrvold* 1979 a).

RESULTS

The experiments showed that red cell recovery was the same whether chromatographed PVP (Table 1), neutralized PVP (Table 2) or untreated PVP (Table 1) was used as the cryoprotective agent. Neutralized PVP was investigated in varying concentrations, and the best result was seen with a final concentration of 10 % similar to that obtained with untreated PVP (*Myhrvold* 1979 a). Lowering the concentration to 7.5 % or raising it to 15 % gave only small differences in cell recovery (Table 2). With PVP at concentrations higher than 10 % the bulk of the loss occurred during the cell washes (Table 2), whereas most of the cells hemolyzed during thawing when starch products (in final concentrations of 7, 10 and 14 %) were used

Table 1. Cryoprotection of sheep red blood cells at —196° using purified polyvinylpyrrolidone[1].

Purification procedure	Loss thawing %	Loss washing %	Recovery %
Sephadex G 25	9.5	4.6	85.9
	11.0	6.9	82.1
Sephadex G 50	8.2	6.2	85.6
	10.5	7.9	81.6
AG 11A8[2]	10.0	4.7	85.3
	12.0	5.9	82.1
	8.1	6.3	85.6
Untreated	9.0	6.3	84.7
	9.2	7.8	83.0

[1] PVP K 15, average molecular weight 10 000.
[2] Ion Retardation Resin (Bio-Rad Laboratories).

Table 2. Cryoprotection of sheep red blood cells at —196° using neutralized polyvinylpyrrolidone.

PVP K 15[1] concentration % (w/v)	Loss thawing %	Loss washing %	Recovery %
5.0	19.3	3.4	77.3
7.5	11.7	3.6	84.7
10	8.6	4.7	86.7
15	8.4	11.3	80.3
20	4.5	25.7	69.8
25	2.8	26.2	71.0
30	25.7	35.1	39.2

[1] Average molecular weight 10 000.

as cryoprotectants (Table 3). Cell recovery was, also shown in Table 3, dependent on type, concentration and time for hydrolysis of the starch used. Best result was seen with hydrolyzed Avelex 1030 and hydrolyzed Avelex 2530 in a final concentration of 14 %. The time for hydrolysis at 100°C should be 40 and 50 min, respectively. These periods were necessary to render the products soluble in the desired concentrations. Cell recovery obtained with Avelex 1030 and Avelex 2530 used as described was the same (80—85 %) as that obtained with the PVP used in these experiments.

Table 3. Cryoprotection of sheep red blood cells at —196° using starch products.

Product	Hydro-lysis[1] min	Final con-centration %	Loss thawing %	Loss washing %	Recovery %
Avelex[2]	40	7	26.8	2.7	70.5
1030	40	10	19.7	1.6	78.7
	40	14	15.7	3.1	81.2
	60	14	31.7	7.2	61.1
	75	14	32.1	17.3	50.6
	150	14	62.4	12.5	25.1
Avelex[2]	50	7	25.9	2.8	71.3
2530	50	10	24.0	2.3	73.7
	50	14	14.0	3.4	82.6
	60	14	26.8	11.3	61.9
	75	14	33.6	7.7	58.7
	150	14	54.5	16.5	29.0
Perfectamyl	40	10	29.5	3.3	67.2
A25/A75	40	14	28.9	4.4	66.7
	60	14	35.7	11.9	52.4
	80	14	40.1	21.7	38.2
Amylogum[4]		7	42.1	3.7	54.2
CL-S		10	32.6	8.0	59.4
		14	41.9	22.3	35.8

[1] At 100°C in 0.15 M-HCl.
[2] Carboxylated potato starches.
[3] Hydroxypropylated potato starch.
[4] A gum product.

DISCUSSION

In this report it was found that the cryoprotective activity of untreated PVP for sheep erythrocytes was the same as with chromatographed or neutralized PVP (80—85 %). Red cell recoveries in the range 80—85 % were also obtained with hydrolyzed Avelex 1030 and hydrolyzed Avelex 2530. Purification of PVP and hydrolyzing of starch were, however, time-consuming. Untreated PVP and neutralized PVP are easily prepared and therefore preferable as cryoprotectants provided the procedure does not lead to any change in the activity of the cells as reagent.

The experiments showed that the total cell loss was comparatively constant, about 15—20 %, independent of the cryoprotectant used. It is possible that a particular fraction of the

cells is rather fragile and also more sensitive to the freezing and thawing procedure. It is conceivable that the removal of these cells will make the rest of the cells less variable when used as reagent.

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SAMMENDRAG

Beskyttelse av røde saueblodlegemer ved frysing. 2. Virkning av renset polyvinylpyrrolidone og hydrolysert stivelse.

Bevaring av røde saueblodlegemer mot skade etter nedfrysing til —196°C ble undersøkt. Som kryoprotektive midler ble brukt renset, nøytralisert og ubehandlet polyvinylpyrrolidone (PVP) og hydrolysert stivelse. God cellegjenvinning ble oppnådd med PVP uavhengig av om denne var renset ved gelfiltrering, om den var nøytralisert eller ubehandlet.

Etter nedfrysing med stivelse var resultatet avhengig av stivelsetype, -konsentrasjon og -hydrolysegrad. Den høyeste gjenvinning med hydrolysert stivelse var 80—85 % og lik den høyeste gjenvinning med PVP.

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