A New Method for Fractionation of Proteins

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Organic solvents and different inorganic salts have frequently been used for fractionation of proteins. The efficiency of organic solvents such as ethanol or ether in decreasing protein solubility has been suggested to be due to the influence of these agents on the dielectric constant of the medium. In the present report it will be shown that amides which are liable to hydrogen bond exchange with proteins can successfully be used for fractional precipitation of proteins.[1] The amides we have used have had the following general formula: $R-CON(R_1R_2)$. Those compounds where R_1 and R_2 are non-polar residues have been shown to be suitable. Such reagents are dimethylformamide (DMFA), N-methylacetamide or N,N-dimethylacetamide among others. It is possible that these reagents precipitate proteins due to hydrogen bonding of the amide with the protein thereby increasing the apolarity of the protein and decreasing its solubility in aqueous media. An alternative explanation would be that the amides exert their action by means of hydrogen exchange with the water of the medium. It might be anticipated that the conditions under which a particular protein is precipitated is to some extent dependent on the amino acid composition of the protein. The ratio between polar and non-polar residues might here be of particular importance.

When an amide such as DMFA is added to blood plasma at neutral pH to a concentration of 8 M practically all of the proteins present in plasma are precipitated. At a concentration of 3.5 M the precipitate formed consists mainly of fibrinogen. At 6 M a considerable part of the γ-globulins is precipitated. Albumin comes out of solution at a higher DMFA concentration. A detailed description of the use of DMFA in fractionation of human plasma proteins is given below. It is, however, to be mentioned, that reagents of this type cannot be used for fractionation of plasma proteins only. With equal success it can be used for fractionation of the proteins of milk.

Precipitation of fraction A I (fibrinogen fraction). To 100 ml of human plasma at 0° is added 37.0 ml of chilled dimethylformamide * (DMFA) to a concentration of 3.5 M. pH is adjusted to 7.0–7.5 with acetic acid. The mixture is equilibrated for 1 h at 0°. The precipitate is removed by centrifugation ** and washed with 3.5 M DMFA in aqueous 0.15 M NaCl (25 ml) at 0°. The recentrifuged precipitate is dissolved in aqueous 0.3 M NaCl and dialysed over night against the same solvent in the cold. Paper electrophoresis of the fraction indicates that it contains mainly fibrinogen (Fig. 1). As judged from the coagulability the fraction consists to about 93 % of fibrinogen (Table 1).

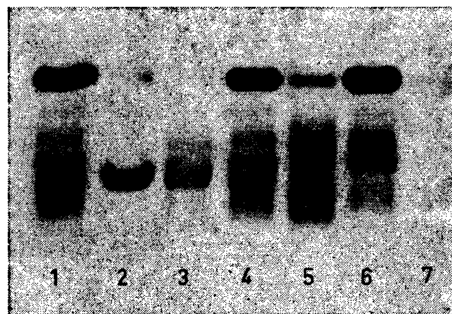

Fig. 1. Electropherogram of plasma and fractions. The electrophoresis was run on cellulose-acetate strips at pH 8.6, 300 V and 4–7 mA for 20 min. The apparatus used was a Beckman model R-101 microzone electrophoresis cell (Beckman instruments, California). A barbital buffer (Beckman B-2), pH 8.6 and ionic strength 0.075 was used. Fractions A I-A III and fraction A V were dissolved in a volume of one fourth and fraction A IV in a volume of one half that of the starting plasma. 1 and 4. Plasma (0.25 μl). 2. Fr. A I (0.25 μl). 3. Fr. A II (0.25 μl). 5. Fr. A III (0.25 μl). 6. Fr. A IV (0.5 μl). 7. Fr. A V (0.25 μl).

* Dimethylformamide (C_3H_7NO); analytical reagent from Mallinckrodt Chemical Works, U.S.A.

** Centrifugation at about 2000 *g*, 0°, for 30–60 min.

Precipitation of fraction A II. To 132 ml of the supernatant of fraction A I at 0° is added 21.3 ml of chilled DMFA to a concentration of 4.8 M. pH is adjusted to between 7 and 7.5 with acetic acid. The mixture is equilibrated for 30 min at 0° and the precipitate formed is removed by centrifugation ** at the same temperature. The precipitate is washed with 4 M DMFA in 0.10 M NaCl (25 ml) at 0°. The orange coloured precipitate is dissolved in 0.15 M NaCl and dialysed as described above. Electrophoresis of the fraction shows the presence of mainly α_2-globulins and globulins with the same mobility as fibrinogen (Fig. 1).

Precipitation of fraction A III. To 137 ml of the supernatant of fraction A II at 0° is added 27.7 ml of chilled DMFA to a concentration of 6.3 M. pH is adjusted to between 7 and 7.5 with acetic acid. The mixture is equilibrated for 30 min at 0° and the precipitate formed is removed by centrifugation ** at the same temperature. The precipitate is washed with 5 M DMFA in 0.09 M NaCl (25 ml) at 0°. The yellowish precipitate is dissolved and dialysed as described above. Electrophoresis of the fraction shows the presence of mainly γ-globulins, α_2- and β-globulins as well as of small amounts of albumin. In addition a band is observed between the α_1- and α_2-globulins (Fig. 1).

Precipitation of A IV. To 155 ml of the supernatant of fraction A III at 0° is added 47.0 ml of chilled DMFA to a concentration of 7.9 M. pH is adjusted to between 7 and 7.5 with acetic acid. The mixture is equilibrated for 30 min at 0° and the precipitate formed is removed by centrifugation ** at the same temperature. The precipitate is washed with 6 M DMFA in 0.08 M NaCl(70 ml) at 0°. The white voluminous precipitate is dissolved and dialysed as described above. Electrophoresis of the fraction shows that it consists predom-

Table 1. Yield of proteins in the different fractions.

Fraction	Protein [b] g	Fibrinogen [c] g	Fibrinogen [c] clottability %	Prothrombin [e] %	Factor IX [f] %	Factor V [g] %	Plasminogen [h] %
Plasma	7.20	0.27	3.7	100.0	100.0	100	100
Washed Fr. A I	0.30	0.21	93.0 [d]	9.0	0.6	0.5	3–4
Wash fluid [a]	0.01	—	—	—	—	—	—
Washed Fr. A II	0.35	0.05	14.3	23.4	12.5	1.0	15–20
Wash fluid [a]	0.29	—	—	—	—	—	—
Washed Fr. A III	1.28	—	—	42.8	41.1	7.0	70–75
Wash fluid [a]	0.75	—	—	—	—	—	—
Washed Fr. A IV	3.31	—	—	9.7	30.0	5.4	6
Wash fluid [a]	0.81	—	—	—	—	—	—
Fr. A V	0.06	—	—	0.1	—	0.6	—

[a] All wash fluids seem, as judged from the electropherograms, mainly to consist of albumin.
[b] Proteins were determined with the method of Lowry *et al.* (Ref. 2) using human albumin (AB Kabi, Stockholm) as standard.
[c] Fibrinogen was determined as clottable protein (with thrombin). The clot was dissolved in urea and the protein concentration determined spectrophotometrically (Ref. 3).
[d] Based on the spectrophotometric analysis (Ref. 3).
[e] Prothrombin was determined in a two-stage assay (Ref. 4).
[f] Factor IX (hemophilia B-factor) was assayed in a recalcification system using hemophilia B plasma as substrate (Ref. 5).
[g] Factor V was determined according to Wolf (Ref. 6).
[h] The determination of plasminogen by a caseinolytic assay (Ref. 7) was kindly performed by Dr. Wallén of this Institute.

inantly of albumin together with α_2- and β-globulins. Weak bands corresponding to the α_1-globulins and to prealbumin are also seen (Fig. 1).

The supernatant, 130 ml, after removal of fraction A IV was dialysed against several changes of water and subsequently freezedried. It is denoted Fraction A V. By electrophoresis it was shown to contain only trace amounts of albumin (Fig. 1).

The yield of proteins in the different fractions is shown in Table 1. In the table is also shown the yield of some specific proteins such as fibrinogen, prothrombin, plasminogen, and hemophilia B-factor (Factor IX). It is evident that as far as these proteins are concerned no irreversible denaturation has occurred. However, only 15 % of the factor V activity of plasma can be recovered in the different fractions (Table 1) which might indicate that this factor has been denatured. Most of the electrophoretic bands demonstrated in the starting plasma can be identified in one or the other of the DMFA-fractions (Fig. 1). The new globulin band observed in fraction A III may have been produced in the course of fractionation and may therefore represent a protein which has been denatured or changed by the amide reagent.

The data of Table 2 show that the ratio of polar/non-polar amino acid residues in the protein might be one of the determining factors for the precipitation of a particular protein. Albumin with a low ratio is precipitated at high DMFA concentrations whereas fibrinogen with a high ratio is precipitated at low concentrations. The proteins with intermediate ratios also keep an intermediate position with respect to precipitability.

The precipitation with DMFA is, as could be expected, not only dependent on the DMFA concentration but also on the pH of the medium. Thus a lower DMFA concentration is needed to precipitate most of the plasma proteins at pH between 5 and 6 than at neutral pH. However, the γ-globulins with isoelectric points of about 7 are more soluble in the former pH-range. It would also be expected that the ionic strength is of importance with respect to the precipitating properties of DMFA.

Table 2. Polar (P) and non-polar (NP) amino acids in some plasma proteins.

Protein	Approximate Mol. Weight	Ratio P/NP	Reference
Albumin	70 000	1.46	*cf.* 8
γ-Globulin	160 000	1.56	*cf.* 8
Prothrombin	70 000	1.63	*cf.* 8
Plasminogen	80 000	1.66	9
Fibrinogen	340 000	1.91	10
		1.91	*cf.* 8, 11

Polar amino acids: Asp, Asn, Glu, Gln, Lys, Arg, Ser, Thr (Ref. 11). Non-polar amino acids: Val, Leu, Ile, Met, Pro, Phe (Ref. 11).

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