Purification of human intrinsic factor using high-performance ion-exchange chromatography as the final step

Jean-Louis Gueant, Ilkka Kouvonen*, Jean-Claude Michalski+, Catherine Masson, Ralph Gräsbeck* and Jean-Pierre Nicolas

*Laboratoire de Biochimie Médicale, Faculté de Médecine, Université de Nancy I, BP 184, 54505 Vandœuvre-les-Nancy Cedex, France, *Minerva Institute for Medical Research, POB 819, 00101 Helsinki 10, Finland* and *+Laboratoire de Chimie Biologique (Laboratoire associé au CNRS n°. 217), 59655 Villeneuve d'Ascq Cedex, France*

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Human intrinsic factor was purified 1430-fold from gastric juice with a yield of 75% using two steps: labile ligand affinity chromatography and high-performance ion-exchange chromatography. Intrinsic factor precipitated in the presence of specific autoantibodies and 15% sodium sulfate, had an estimated M_r of 59000 in 5% SDS electrophoresis and could bind to the specific ileal receptor in vitro. Its carbohydrate composition could be related to *N*-lactosaminic and *O*-glycosidic chains. High-performance ion-exchange chromatography was a mild, rapid and efficient procedure to separate completely intrinsic factor from haptocorrin (another glycoprotein of gastric juice which binds cobalamin) and from other contaminating proteins.

Intrinsic factor *Cobalamin* *Vitamin B12 binding protein* *High-performance liquid chromatography* *Ion-exchange chromatography* *Glycoprotein*

1. INTRODUCTION

In man, the intraluminal transport of cobalamin (vitamin B12) involves 2 glycoproteins, intrinsic factor (IF) and haptocorrin (Hc, also named R protein or R binder). These two binders are present in gastric juice. IF is a permease which promotes the uptake of dietary cobalamin (Cbl) in the distal ileum via specific receptors. The binding of cobalamin to IF is very specific while haptocorrin binds numerous cobalamin analogues (e.g., cobinamide).

Affinity chromatography is mostly used in the purification of IF as an initial or as a single step, with the exception of a recent work which used immunoadsorption to anti-IF autoantibodies [1]. This type of affinity chromatography uses cobalamin bound to amino-Sepharose by a temperature-sensitive Co-N or a light-sensitive Co-C linkage [2,3]. In other procedures, a cobalamin derivative is covalently coupled to amino-substituted Sepharose [4,5]. In these cases, the desorption is provided by a denaturation of the Cbl binder by agents such as guanidine hydrochloride which may modify the structure and composition of the molecule [6,7]. Another step of the purification is needed to separate IF from haptocorrin and other proteic material. Several procedures are used, e.g., gel filtration [2] or affinity chromatography with immobilized corrinoids [8]. Here, we have purified intrinsic factor from human gastric juice using two steps: labile ligand affinity chromatography and high-performance ion-exchange chromatography (HPIEC).

2. MATERIALS AND METHODS

2.1. *Chemicals*

Crystalline hydroxocobalamin (OH-Cbl) was obtained from Sigma, St Louis, crystalline

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cobinamide from Calbiochem, La Jolla and cyano[^{57}Co]cobalamin (spec. act. 220 μCi/μg) from the Radiochemical Centre, Amersham. Activated AH-Sepharose-4B was purchased from Pharmacia, Uppsala, Sweden. All other reagents were obtained as described previously [9].

2.2. *Gastric juice*

Normal human gastric juice was aspirated, after pentagastrin stimulation, by naso-gastric tube. The pepsin activity was reduced by consecutive pH adjustment to 10.0 and 7.0 as described [10]. The pooled gastric juice was stored at −20°C. It was filtered through a Celite layer in a Büchner funnel followed by centrifugation at 17000 × *g* for 30 min. Unsaturated Cbl binding capacities of IF and haptocorrin were determined using the methods of Gottlieb et al. [11] and Begley and Trachtenberg [12] with hemoglobin-coated charcoal and cobinamide. Haptocorrin was saturated by incubation of gastric juice with cobinamide (400-fold the cobalamin binding capacity). Gastric juice was mixed with 0.1 M Tris-HCl buffer (pH 8.0) (10% of the total volume).

2.3. *Affinity chromatography*

Hydroxocobalamin was coupled to activated AH-Sepharose-4B by a thermolabile Co-N linkage according to the method of Nexø [2]. Hydroxo[^{57}Co]cobalamin (OH-[^{57}Co]Cbl) in 0.1 M $NaHCO_3$ was prepared from cyano[^{57}Co]cobalamin by light exposure in 8 mM HCl. 4 μmol OH-Cbl and 13 pmol OH-[^{57}Co]Cbl were incubated with 4 g Swollen AH-Sepharose-4B in 0.1 M $NaHCO_3$ (pH 8.2) containing 0.5 M NaCl at 4°C for 24 h under rotative agitation and protection from light. The suspension was subsequently poured onto a column and washed with 500 ml of 0.1 M $NaHCo_3$ (pH 8.2) containing 0.5 M NaCl, 500 ml of 0.5 M NaCl and 500 ml of 0.02 M Tris-HCl buffer (pH 8.0). The coupling efficiencies of OH-Cbl and OH-[^{57}Co]Cbl, determined using spectrophotometry and γ-ray counting (Packard Autogamma, model 4780), were about 20 and 14%, respectively. After the gastric juice was applied to the column, the gel was washed successively with 250 ml of 0.02 M Tris-HCl buffer (pH 8.2) containing 0.5 M NaCl and 250 ml of 0.02 M Tris-HCl buffer (pH 8.4). The total amount of IF coupled to the unsolubilized Cbl was estimated from determination of unsaturated cobalamin binding capacity of gastric juice before and after elution. After incubation of the column at 37°C for 20 h, the cobalamin intrinsic factor complexes were removed by washing the column with 20 ml of 0.02 M Tris-HCl buffer (pH 8.4) or 0.05 M sodium phosphate buffer (pH 7.4) at room temperature.

2.4. *High-performance ion-exchange chromatography*

HPIEC was carried out with an analytical HPLC system (Waters Associates, Milford, USA). The sample was dialyzed and concentrated up to 2 ml by ultrafiltration using a YM-10 Diaflo membrane (Amicon, USA) and poured onto an anionic exchange column (Mono Q, Pharmacia). The mobile phase was 0.02 M Tris-HCl buffer (pH 8.4) with a 0–100% NaCl gradient at a flow rate of 1.0 ml·min^{-1}. The proteins and the cobalamin binders were detected by spectrophotometry at 280 nm and radioactivity counting, respectively. The effluent was collected in 0.2-ml fractions (frac 300, Pharmacia).

2.5. *Gel filtration*

Part of the sample obtained from affinity chromatography was filtered through a Sephadex G-200 column (2.5 × 90 cm) in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 3 mmol/l NaN_3 according to the method of Nexø [2].

2.6. *Electrophoresis*

Polyacrylamide disc gel electrophoresis was carried out according to Fairbanks et al. [13]. The sample contained 20 μg proteins and 10% sucrose (w/v) in a total volume of 40 μl. The gels were stained for protein with Coomassie brilliant blue. The M_r of IF was estimated in 5% SDS-polyacrylamide electrophoresis using lactalbumin, soybean trypsin inhibitor, ovalbumin, bovine serum albumin and phosphorylase *b* as reference molecules.

2.7. *Amino acid and carbohydrate analysis*

A part of the sample obtained from HPIEC was dialyzed, concentrated on a YM-10 ultrafiltration membrane and lyophilized for amino acid and carbohydrate analysis. The amino acid content of IF

was determined after hydrolysis of the sample (0.17–0.25 mg) in a Beckman amino acid analyzer. The molar carbohydrate composition of the glycoprotein was determined according to the method of Zanetta et al. [14] modified as follows: 150 μg glycoprotein (about 20 μg carbohydrates) was subjected to acid methanolysis in 1 ml MeOH containing 0.5 M HCl for 24 h at 80°C. The sample was cooled to room temperature and the methanolysate was extracted twice with 1 ml hexane. The upper phases containing mostly amino acids were discarded and the lower phases were concentrated under a nitrogen flow. The residue was treated with 10 μl of dichloromethane and 10 μl of trifluoroacetic anhydride for 12 h at room temperature. The carbohydrate derivatives were identified by gas chromatography.

2.8. *Activity of IF*

The immunoreactivity of the purified IF-[^{57}Co]Cbl complex was tested by incubating the sample with a pernicious anaemia serum rich in binding-type anti-IF autoantibodies and by precipitating the immunocomplexes in 15% (w/v) sodium sulfate. A sample of the purified IF-[^{57}Co]Cbl was incubated with the receptor extract prepared from pig small intestine as described earlier [15,16]. Pig IF-receptor is known to react with human IF. The incubation was performed in the presence of 0.1 mg $CaCl_2$ (in 2 ml) or 5 μmol/l of EDTA. Each incubation mixture was then run through a Sephadex G-200 column (2.5 × 90 cm) in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 mol/l NaCl, 0.01% merthiolate and 0.05% Triton X-100.

3. RESULTS

IF has been purified 1430-fold with a yield of 75% (table 1). It represented about 10% of the total unsaturated cobalamin binding capacity of gastric juice. 85% of the unsaturated cobalamin binding capacity of the treated gastric juice was immobilized onto the affinity column. Only some traces of radioactivity were found in the gel after its thermosensitive desorption. In HPIEC, the single collected radioactive peak was also the first eluted peak detected at 280 nm (fig.1). It corresponded to saturated Cbl binders. Acidic proteins devoid of Cbl binding capacity were removed at a higher ionic strength. In another experiment, the use of guanidine hydrochloride during the desorption of the affinity column did not modify the liberation of these proteins. The radioactive peak collected from HPIEC was desalted and concentrated by ultrafiltration. The saturated Cbl binder presented the characteristic UV absorption spectrum of an IF-Cbl complex [5,18]. The purified IF moved as a single protein band in polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. In the latter, the migration corresponded to an estimated M_r of about 59000 (fig.2). The amino acid composition of the molecule was similar to those previously determined [5,18] (table 2). The carbohydrates extracted from the sample of purified IF corresponded to 14.2% of the molecule. The carbohydrate content of the purified IF is indicated in table 2. When IF was incubated with type II anti-IF autoantibodies, more than 98% of the [^{57}Co]Cbl-IF precipitated in the presence of 15% sodium sulfate

Table 1

Purification of human intrinsic factor (IF)

	Volume (ml)	IF-Cbl binding capacity (nmol)	Protein content (mg)	Specific activity (nmol/mg)	Purification factor	Yield (%)
Human gastric juice	1450	48.1	3045	0.0158	–	–
Affinity chromatography on Cbl Sepharose	17	40.6[a]	15.4	2.6	164	83.2
HPIEC	4.5	36.1[a]	1.8	22.6	1430	75.0

[a] Determined from absorbance at 360 nm [17]

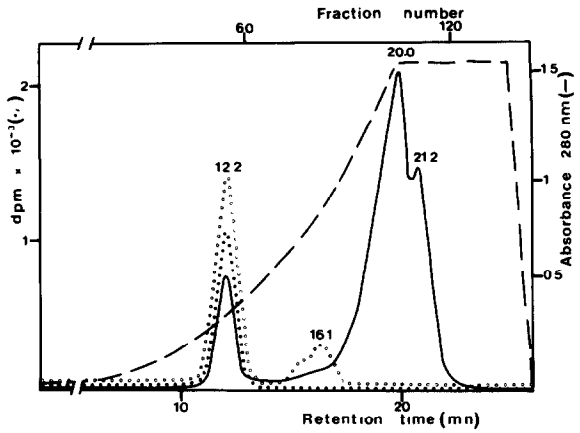

Fig.1. Elution profile of the affinity chromatography eluate (500 μl) on HPIEC (column Mono Q). The mobile phase was 0.02 M Tris-HCl buffer (pH 8.4) with a gradient of 0–1 M NaCl in 15 ml. Flow rate 1 ml·h^{-1}, detection by absorbance at 280 nm (——) and radioactivity of [^{57}Co]Cbl. When the gastric juice was previously incubated with cobinamide, a single radioactive peak was detected with a retention time of 12.2 (···) corresponding to intrinsic factor. When cobinamide was not added, Hc was also detected with a retention time of 16.1 (ooo).

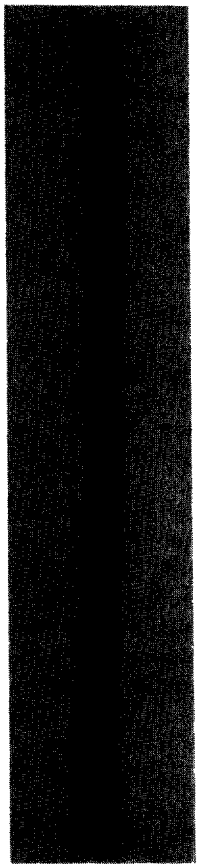

Fig.2. SDS-polyacrylamide gel electrophoresis of 20 μg human intrinsic factor. A single band was identified with an R_f of 0.23 corresponding to an M_r of 59000.

(w/v) corresponding to the formation of a specific immune complex. The IF-[^{57}Co]Cbl complex bound to the ileal specific receptor in the presence of $CaCl_2$ because the filtration of the incubation mixture through a Sephadex G-200 column showed the elution of the complex in the void volume fractions. In the control experiment in the presence of EDTA the receptor complex was not able to form, since no peak emerged in the void volume of the gel filtration. When part of the sample of semi-purified IF obtained after desorption of the affinity gel was filtered through a Sephadex G-200 column, a single symmetrical radioactive peak was collected, corresponding to IF-[^{57}Co]Cbl. This peak had an A_{280}/A_{360} ratio of about 3.5. This high ratio can be explained by the presence of some contaminating protein material devoid of Cbl binding capacity. In other purification experiments, we obtained a lower ratio, which could be explained by differences in the initial protein composition of gastric juice.

4. DISCUSSION

Several authors have purified unsaturated Cbl binders using elution of the affinity column with guanidine hydrochloride [5,6]. This procedure induces a strong denaturation of the proteins [6] even if it is carried out in presence of free cobalamin. Moreover, it is not certain that the changes of the molecule are completely reversed during subsequent dialysis. However, unsaturated IF can be separated from Hc in a second step using another affinity chromatography elution. Labile ligand affinity chromatography is a very mild procedure. The semi-purified molecule is then saturated with OH-Cbl, and it is therefore impossible to achieve the purification using affinity chromatography in a second step. Gel filtration

Table 2

Amino acid content (residues per mol) of human intrinsic factor

Amino acid	Present study	Allen and Mehlman [5]	Nexø et al. [18]	Christensen et al. [4]
Lysine	18	20	19	16
Histidine	5	5	4	5
Arginine	6	6	5	6
Aspartic acid	39	38	39	37
Threonine	24	24	26	26
Serine	31	30	36	33
Glutamic acid	36	35	33	36
Proline	23	22	21	32
Glycine	23	20	20	24
Alanine	24	23	24	23
Valine	23	22	24	24
Ileucine	20	22	23	20
Leucine	30	34	35	30
Tyrosine	9	9	10	10
Phenylalanine	9	9	10	10
Methionine	10	10	11	8
Half-cystine	5	6	6	12
Tryptophan	6	6	3	–
Total	344	342	349	351

has been previously used for this purpose [2]. In our experience, this technique does not always remove the proteins present in the IF sample after the affinity chromatography. These proteins could be adsorbed to the arm of the affinity gel by unspecific interactions during the elution of gastric juice. Desorption of the gel with variable ionic strength buffers or with agents as guanidine hydrochloride does not modify the removal of these proteins. They can be removed easily from IF using HPIEC (fig.1). A second problem in the purification of IF is to eliminate completely Hc in the final sample. Here, we saturated the unsaturated cobalamin binding capacity of Hc in gastric juice using cobinamide. In another experiment, we separated partially degraded and undegraded Hc from IF in the HPIEC step of the purification. Hc is more acidic than IF and is eluted at a higher ionic strength (fig.1). Each peak was identified by specific immunoprecipitation in 15% sodium sulfate. The purified IF had similar physicochemical characteristics to those previously described in respect to its electrophoretic mobility, UV absorption spectra and amino acid composition. Gel filtration has also been employed by several authors to separate Hc from IF in a final step of purification [2]. In our experience, gel filtration cannot separate IF from partially degraded Hc which is present in the gastric juice, since both molecules have similar molecular masses [19]. The amino acid composition of IF was similar to those previously determined [4,5,18] whereas significant differences were observed in the car-

Table 3

Carbohydrate content of human intrinsic factor

	mol per 3 mol mannose[a]
Fucose	3.22 (1.75)
Galactose	6.06 (1.50)
Mannose	3.00 (3.00)
Glucose	1.10 (0.00)
N-Acetylgalactosamine	2.71 (0.75)
N-Acetylglucosamine	5.21 (1.50)
Sialic acid	1.99 (0.75)

[a] Values in parentheses calculated from [5]

bohydrate composition, particularly concerning the number of mannose residues (table 3). The carbohydrate content of IF, according to several authors [2,5], corresponded to a glycoprotein with polymannosidic chains [20]. With regard to our results, it may be assumed that IF contains mainly *N*-lactosaminic-type carbohydrate chains [20]. Moreover, the presence of Gal-Nac residues indicated the presence of *O*-glycosidic carbohydrate chains on the molecule. Another predominant feature is the high content of fucose, in comparison to sialic acid. This observation may be related to the biosynthetic origin of the glycoprotein. Fucose may also be related to a structural recognition for the final destination of the glycoprotein. It must be noted that gels such as Sephadex or Sepharose in the purification steps may dissociate glucose which becomes a contaminant in gas chromatography. Glucose can be detected as mannose residue since it may have a very similar retention time in gas chromatography.

In conclusion, the sequence of labile ligand affinity chromatography and HPIEC seems to be an efficient and mild procedure to purified human IF since (i) the purified molecule is not treated using denaturing agents, and (ii) the second step can be completed very quickly and completely removes Hc and other contaminating acidic proteins from IF.

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REFERENCES

[1] Shephard, N.A., Priddle, J.D., Jenkins, W.J. and Jewell, D.P. (1984) Clin. Chim. Acta 139, 155–165.
[2] Nexø, E. (1975) Biochim. Biophys. Acta 379, 189–192.
[3] Jacobsen, D.W. and Montejano, Y. (1979) in: Vitamin B12, pp.949–952, De Gruyter, Berlin.
[4] Christensen, J.M., Hippe, E., Olesen, H., Rye, M., Haber, E., Lee, L. and Thomsen, J. (1973) Biochim. Biophys. Acta 303, 319–332.
[5] Allen, R.H. and Mehlman, C.S. (1973) J. Biol. Chem. 248, 3660–3669.
[6] Weiss, J.P., Rothenberg, S.P. and Cotter, R. (1977) FEBS Lett. 78, 275–277.
[7] Stenman, U.H. (1975) Scand. J. Clin. Lab. Invest. 35, 147–155.
[8] Allen, R.H. and Mehlman, C.S. (1973) J. Biol. Chem. 248, 3670–3680.
[9] Guéant, J.L., Vidailhet, M., Pasquet, C., Djalali, M. and Nicolas, J.P. (1984) Clin. Chim. Acta 137, 33–41.
[10] Gräsbeck, R. (1956) Acta Med. Scand. 154, 780–784.
[11] Gottlieb, C.W., Retief, F.P. and Herbert, V. (1967) Biochim. Biophys. Acta 141, 560–567.
[12] Begley, J. and Trachtenberg, A. (1979) Blood 53, 788–793.
[13] Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.
[14] Zanetta, J.P., Breckenridge, W.C. and Vincendon, G. (1972) J. Chromatogr. 69, 291–301.
[15] Kouvonen, I. and Gräsbeck, R. (1979) Biochem. Biophys. Res. Commun. 86, 358–364.
[16] Kouvonen, I. and Gräsbeck, R. (1981) J. Biol. Chem. 256, 154–158.
[17] Nexø, E. and Olesen, H. (1976) Biochim. Biophys. Acta 446, 143.
[18] Nexø, E., Olesen, H., Hansen, M.R., Bucher, D. and Thomsen, J. (1978) Scand. J. Clin. Lab. Invest. 38, 649–653.
[19] Guéant, J.L., Parmentier, Y., Djalali, M., Bois, F. and Nicolas, J.P. (1983) Clin. Chim. Acta 134, 95–106.
[20] Montreuil, J. (1982) in: Comprehensive Biochemistry (Neuberger, A. and Van Deenen, L. eds) vol.19B, part II, pp.1–188, Elsevier, Amsterdam, Oxford, New York.