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# Chromatographic separation of low-molecular-mass recombinant proteins and peptides on Superdex 30 prep grade

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**Abstract**

The chromatographic properties of Superdex 30 prep grade medium have been investigated in non-denaturing and denaturing mobile phases using commercially available proteins and peptides as well as low-molecular-mass ($M_r$) recombinant polypeptides. The medium is a macroreticular gel composed of crosslinked agarose beads to which dextran has been covalently bound. The mean particle size is approximately 34 $\mu$m. Experimental results show a linear relation between the distribution coefficient ($K_D$) and the $\log_{10} M_r$ in the fractionation range 24 000–3000. The relationships between resolution and flow-rate or load volume were investigated and shown to be comparable with those of Superdex 75 and 200 prep grade media. Minimal loss of resolution occurred in the flow-range from 30–60 cm/h. Load volumes of up to 5% total column volume could be applied while maintaining baseline resolution of polypeptide mixtures. Non-specific interactions between the matrix and certain samples were characterized. The predominant interactions with the resin appear to be hydrophobic in nature rather than ionic. Hydrogen bonding may also play a role in the retardation of certain small molecules. The applicability of the resin for separating dimeric and oligomeric forms of low-molecular-mass recombinant proteins was shown.

## 1. Introduction

The recent introduction of Superdex 30, 75, and 200 preparative grade media for the aqueous gel permeation of biomolecules has provided matrices which combine the high degree of selectivity of dextrans with the low flow resistance of macroreticular gels [1]. This is accomplished through the covalent incorporation of dextran into crosslinked agarose beads. Such media possess a high degree of chemical and physical stability, enabling them to be operated at sufficiently high flow-rates to allow for efficient large-scale processing, yet are capable of high resolution as well. The fractionation ranges for Superdex 75 and 200 prep grade media are 70 000–3000 and 600 000–10 000 $M_r$, respectively, and both are operated at flow-rates up to 50 cm/h [1]. Recently, Superdex 30 has become available for fractionating smaller proteins and larger peptides; the fractionation range as given in the product data sheet is up to $M_r$ 10 000 for polypeptides.

Many small proteins and large peptides possessing antithrombotic activity have been isolated from hematophagous organisms. These products represent drug candidates or lead compounds through their inhibition of blood coagulation factors or platelet aggregation [2–5]. Tick anticoagulant peptide (TAP) is a 60-amino-acid peptide ($M_r$ 6977, p*I* 4.9), found in the saliva of the soft tick *Ornithodorous moubata* that specifically

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inhibits blood coagulation factor Xa [2]. Hirudin (HIR) is a family of single polypeptide variants of 64–66 amino acids originally isolated from the medicinal leech, *Hirudo medicinalis* and is a highly specific and potent inhibitor of thrombin [3]. The salivary glands of the leech *Haementeria officinalis* contain a protein, leech antiplatelet protein (LAPP), that specifically blocks collagen-mediated platelet aggregation [4]. Echistatin is a 49-residue protein purified from the venom of the saw-scaled viper, *Echis carinatus*, that inhibits platelet aggregation [5].

In addition to being isolated from native sources, many of these polypeptides have been expressed as secreted proteins in recombinant yeast cultures and subsequently purified in order to obtain sufficient amounts for *in vivo* and *in vitro* evaluations [6–9]. Also, a mutant form of recombinant TAP (rTAP), (TAP Y1W/D10R), containing the amino acid substitutions $Y_1 \rightarrow W_1$ and $D_{10} \rightarrow R_{10}$, has been expressed and purified (J. Cook, manuscript in preparation). The need to purify and characterize biomolecules in this $M_r$-range makes a gel filtration medium such as Superdex 30 useful. This report presents an evaluation of Superdex 30 for fractionating proteins and peptides in the $M_r$ range of 43 000–1000.

## 2. Experimental

### 2.1. Equipment

Chromatography was performed on a Pharmacia FPLC system consisting of a P500 high-precision pump and controller, M7 motor valve equipped with sample loop, and a Frac 200 fraction collector. A Waters 990 photo-diode array detector and associated software were used to monitor absorbance and to perform chromatographic analysis.

### 2.2. Chemicals and reagents

Superdex 30 prep grade resin was obtained from Pharmacia (Uppsala, Sweden) pre-packed in a glass XK16 column (600 × 16 mm I.D.). Table 1 lists the sources of the various proteins and peptides used in this study. Recombinant hirudin (rHIR) [6], recombinant LAPP (rLAPP) [E.D. Lehman, unpublished results], rTAP [7], recombinant echistatin (r-echistatin) [9], and rTAP (Y1W/D10R) [J. Cook, manuscript in preparation] were produced as secreted products in an *S. cerevisiae* expression system and were purified in our laboratory. These preparations were ≥99% pure by reversed-phase HPLC analysis and were homogeneous by limited $NH_2$-terminal sequencing. Synthetic echistatin was a gift from Dr. V. Garsky (Merck Research Laboratories) and was prepared as previously described [10]. The amino acids L-tryptophan, L-phenylalanine, and L-tyrosine were obtained from Pierce (Rockford, IL, USA). 3-Indole propionic acid and 4-vinylpyridine were purchased from Aldrich (Milwaukee, WI, USA). N-α-Methyl-DL-tryptophan was purchased from Chemalog (South Plainfield, NJ, USA). Glutathione and pepstatin A were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent-grade purity or better. Proteins and peptides were dissolved in water or buffer at 2–10 mg/ml. All subsequent dilutions were made in mobile phase as described in the respective figures.

### 2.3. Chromatographic and chemical methods

Column preservative (24% ethanol) was removed by washing with 5 column volumes Milli-Q-water followed by equilibration into mobile phase for 5 column volumes. Reduction and alkylation of proteins was carried out as described by Friedman *et al.* [11]. A 100-fold molar excess of 2-mercaptoethanol over total disulfides was added and incubation proceeded under nitrogen for 2 h at 37°C. A 1:1 molar ratio of 4-vinylpyridine (4-VP) to total free sulfhydryl groups was added and incubation proceeded under nitrogen for 2 h at room temperature. Excess reagent was removed by desalting on an Econopak 10DG column obtained from Bio-Rad.

Table 1
Data for proteins and peptides fractionated on Superdex 30

| Sample | | Source[a] | Molecular mass | Efficiency ($N$) | Recovery[b] (%) |
|---|---|---|---|---|---|
| No. | Name | | | | |
| 1 | Thyroglobulin | A | 669 000 | 3760 | N.D.[c] |
| 2 | Ovalbumin | A | 43 000 | 3500 | N.D. |
| 3 | Chymotrypsinogen A | A | 23 650 | 2840 | N.D. |
| 4 | Myoglobin | A | 17 000 | 2429 | 90 |
| 5 | Lysozyme | B | 14 000 | 1809 | N.D. |
| 6 | rLAPP | C | 13 642 | 2369 | 83 |
| 7 | Cytochrome c | A | 12 400 | 2607 | N.D. |
| 8 | rTAP | C | 6977 | 2610 | 94 |
| 9 | rHirudin | C | 6892 | 2494 | 42 |
| 10 | Arg-insulin | A | 5964 | 560 | 92 |
| 11 | Echistatin | C | 5413 | 2947 | N.D. |
| 12 | Poly-L-lysine | A | 4000 | N.D. | N.D. |
| 13 | Insulin B chain-2($SO_3$) | A | 3496 | 2844 | N.D. |
| [d] | $\alpha_1$-Mating Factor | A | 1918 | N.D. | 75 |
| 14 | Bombesin | A | 1620 | 4352 | 92 |
| 15 | Substance P | A | 1552 | 3155 | 94 |
| 16 | Oxytocin | A | 1007 | 4169 | 54 |
| [d] | Tryptophan | E | 186 | 6716 | N.D. |
| 17 | $NaNO_3$ | D | 85 | 7865 | N.D. |

Chromatography was performed using a mobile phase of 6.25 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Flow-rate was 30 cm/h. Injection volume was 0.8% $V_c$ (1.0 ml). Detection was by absorbance at 280 nm or 220 nm.

[a] (A) Sigma (St. Louis, MO, USA); (B) Worthington Diagnostics (Freehold, NJ, USA); (C) Merck Research Laboratories (West Point, PA, USA); (D) Merck & Co. (Rahway, NJ, USA); (E) Pierce (Rockford, IL, USA).

[b] Calculated from the absorbance at 280 nm or 220 nm of the pooled eluate as compared to a dilution of the load material at the same volume.

[c] N.D.: not determined.

[d] Not included in Fig. 1.

### 2.4. Calculations

The distribution coefficient ($K_D$) for a protein was calculated by the equation:

$$K_D = (V_R - V_0)/(V_T - V_0)$$

where $V_R$ is the retention volume (ml) of the polypeptide, $V_0$ is the void volume (ml) of the column as determined by the $V_R$ of thyroglobulin, and $V_T$ is the total liquid volume (ml) of the column as determined by the $V_R$ of $NaNO_3$.

Column efficiency expressed by the number of theoretical plates ($N$) was calculated by the equation:

$$N = 5.54\ (V_R/W_{1/2})^2$$

where $W_{1/2}$ is the peak width measured at half-height.

Resolution ($R_S$) was calculated from the retention volume ($V_{Rn}$) and the width at baseline ($W_n$) of each peak by the equation:

$$R_S = 2(V_{R,n-1} - V_{R,n})/(W_{n-1} + W_n)$$

## 3. Results and discussion

### 3.1. Column characterization and performance

We have evaluated the chromatographic properties of Superdex 30 prep grade medium with several commercially available proteins and pep-

tides, the synthetic polypeptide echistatin, as well as the recombinant proteins rTAP, rTAP (Y1W/D10R), rHIR, and rLAPP that were purified in our laboratories. Table 1 lists the sources of the various proteins and peptides used in this study. Recoveries of proteins and peptides were assessed by comparing the absorbance at 280 nm or 220 nm of the polypeptide eluted from the column with that of the charge to the column. Recoveries were greater than 80% for most samples, except for rHIR, oxytocin and α1-mating factor, which gave recoveries of <80%. The polypeptides giving lower recoveries may be exhibiting some interactions with the gel.

Column efficiency ($N$) is given in Table 1 for molecules of various $M_r$. Experimental conditions are indicated in the table. The highest efficiency, 7865, was obtained with the totally permeating salt $NaNO_3$. Proteins and peptides yielded values from 560 to 4352. Small molecules ($M_r < 2000$) gave higher efficiencies than polypeptides with molecular masses within the gel fractionation range, because the larger molecules exhibit slower mass transport due to their smaller diffusion coefficients. Larger proteins such as thyroglobulin and ovalbumin showed higher efficiencies than predicted from their diffusion coefficients because they are completely excluded from the pores of the matrix. rLAPP ($M_r = 13\,642$) gave a column efficiency of 2369, which was comparable to that of myoglobin ($M_r = 17\,000$), which gave $N = 2429$. However, the $K_D$ of rLAPP indicated an $M_r$ of 20 000. Little is known of the three-dimensional structure of rLAPP, but its behavior during gel permeation might indicate that it has a shape more elongated than globular, a looser structure, or that some interaction(s) with the column matrix is occurring. Other data (see below) have ruled out the possibility of oligomeric forms. Chymotrypsinogen A gave an efficiency that was unexpectedly high for its size.

The resolution properties of the gel are shown in Fig. 1, in which the relationship between $M_r$ of peptides and proteins and the distribution coefficient, $K_D$, is plotted. The samples ranged in $M_r$ from 669 000 (thyroglobulin) to 1007 (oxytocin); 250 μg of each sample was chromatographed. The total volume of the column was marked with 1% $NaNO_3$. The selectivity curve was typically sigmoidal and the linear resolution range was 3000 − 24 000 ($K_D$ from 0.08 to 0.42), with a slope of $-0.40\ K_D \cdot \log_{10} M_r^{-1}$. However, the practical fractionation range of the gel is greater than the linear range, as proteins in the molecular mass range of 43 500 to 1007 eluted between the $V_0$ and $V_T$. Deviations from linearity exhibited by peptides with a $M_r$ below 3000 can be primarily attributed to two factors. Since migration of molecules in a gel permeation medium is dependent not only on the absolute mass but also upon molecular shape and hydrodynamic radius, smaller peptides with little secondary and tertiary structure do not tend to fall on the same selectivity curve as more compact, globular proteins. As such, $M_r$ estimation with Superdex 30 is reliable only if the structure of the molecule whose $M_r$ is to be determined is known to be similar to that of the calibration standards. A second factor affecting migration is interaction(s) with the matrix.

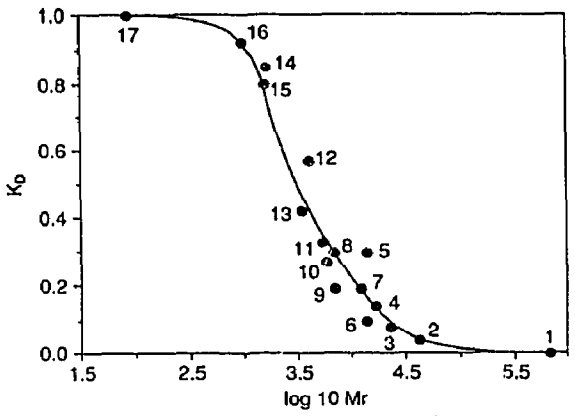


Fig. 1. $K_D$ of polypeptides *vs.* $\log_{10} M_r$. Injection of thyroglobulin was used to determine $V_0$ and 1% $NaNO_3$ was used to determine $V_T$. Sample numbers and experimental conditions are given in Table 1.

The resolution properties of the gel are also represented by a typical chromatogram of a polypeptide mixture shown in Fig. 2. Polypeptides with a difference in $M_r$ of a factor as little as 1.2 (rTAP and echistatin) were successfully resolved. This is in agreement with conclusions reached from a theoretical point of view [12].

The effect of flow-rate on column efficiency is

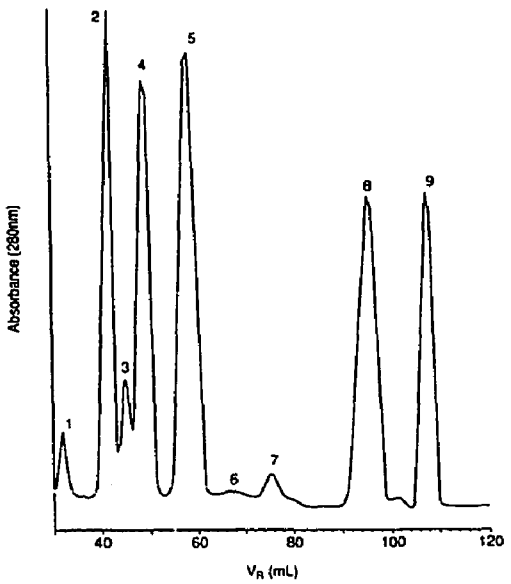


Fig. 2. Chromatographic resolution of a polypeptide test mixture. Flow-rate was 30 cm/h. Detection was by absorbance at 280 nm. Injection volume was 0.8% $V_c$ (1.0 ml). Each polypeptide was present at 0.25 mg/ml. Concentration of $NaNO_3$ was 1%. Peaks: 1 = thyroglobulin, 2 = α-chymotrypsinogen A, 3 = myoglobin, 4 = cytochrome c, 5 = rTAP, 6 = echistatin, 7 = Insulin B chain-2($SO_3$), 8 = bombesin, 9 = $NaNO_3$.

illustrated in Fig. 3. Fixed-volume injections of a solution of rTAP were made at various flow-rates and $N$ was calculated for each run. Efficiency decreased more quickly at very low flow-rates but above 15 cm/h the rate of decline was more gradual. This characteristic has been reported for Superdex 75 and 200 prep grade media [1]. Typical operational flow-rates for this column would be 30–60 cm/h.

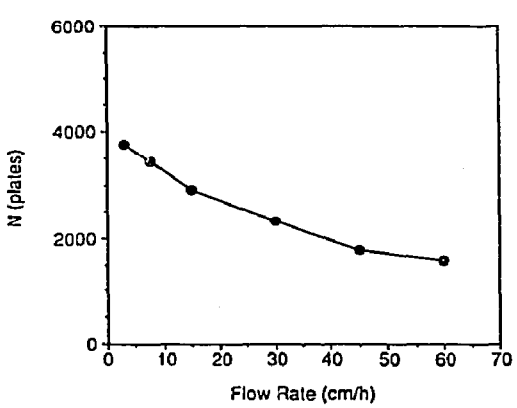


Fig. 3. Plate number ($N$) *vs.* flow-rate. The sample was rTAP (0.75 mg/ml). An injection volume of 0.8% $V_c$ (1.0 ml) was used. Detection was by absorbance at 280 nm. Other conditions as in Table 1.

The relationship between resolution ($R_s$) and the load volume is illustrated in Fig. 4. Mixtures of rTAP and myoglobin, proteins with a $M_r$ difference factor of 2.4, were made at a fixed concentration of each protein. Injections of increasing volume were made at a flow-rate of 30 cm/h. The experiment was repeated using a mixture of rTAP and cytochrome c, $M_r$ difference factor of 1.8, at the same concentrations and injection volumes. At load volumes up to 3% of the geometric column volume ($V_c$), rTAP and myoglobin were resolved at the baseline ($R_S \geq 1.5$). The resolution between rTAP and cytochrome c decreased more quickly. At loading of 1.6% of $V_c$, the proteins were no longer

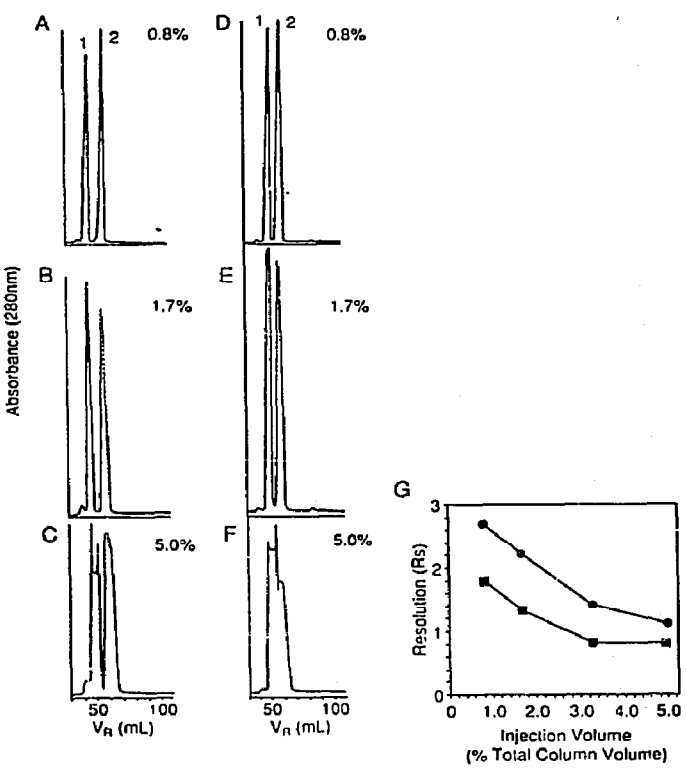


Fig. 4. Influence of sample volume (as % geometric column volume) on $R_S$. Panels A–C represent the myoglobin(1)/rTAP(2) pair and panels D–F represent the cytochrome C(1)/rTAP(2) pair. Protein concentration was 3.0 mg/ml. Detection was by absorbance at 280 nm. Other conditions as in Table 1. For G, (●) myoglobin/r-TAP and (■) cytochrome c/r-TAP.

resolved at the baseline. It would be expected that baseline resolution should be lost at a lower load in the rTAP/cytochrome c case since the $M_r$ difference factor is smaller than for rTAP/myoglobin. The comparison of the resolution of proteins of different $M_r$ difference factors at different sample volumes could probably be explained by taking into account the differences in the steepness of the selectivity curve and the diffusion coefficients of the proteins, rather than some inherent property of the matrix. Superdex 30 prep grade medium provided higher resolution at greater loading volumes than Superdex 200 prep grade. The latter exhibited a resolution of $<1.5$ at $\leq 0.5\%$ of $V_c$ when separating proteins of similar $M_r$ difference factors (1.9). Superdex 30 was more comparable to Superdex 75 which provided baseline resolution ($>1.5$) at $\leq 3.0\%$ $V_c$ [1] when separating similarly sized samples.

The effect of flow-rate on resolution is illustrated in Fig. 5. A mixture of rTAP and cytochrome c at constant concentration and fixed injection volume was chromatographed at various flow-rates. As with efficiency, an initial drop in $R_S$ occurred at flow-rates up to 15 cm/h, an effect also observed with Superdex 200 and 75 prep grade media [1]. From 15 cm/h to 60 cm/h $R_S$ decreased more gradually. At 60 cm/h, baseline resolution was still observed. These results imply that processing time may be decreased by increasing the flow-rate from 15 cm/h to 60 cm/h without seriously impeding the ability to resolve two closely eluting species.

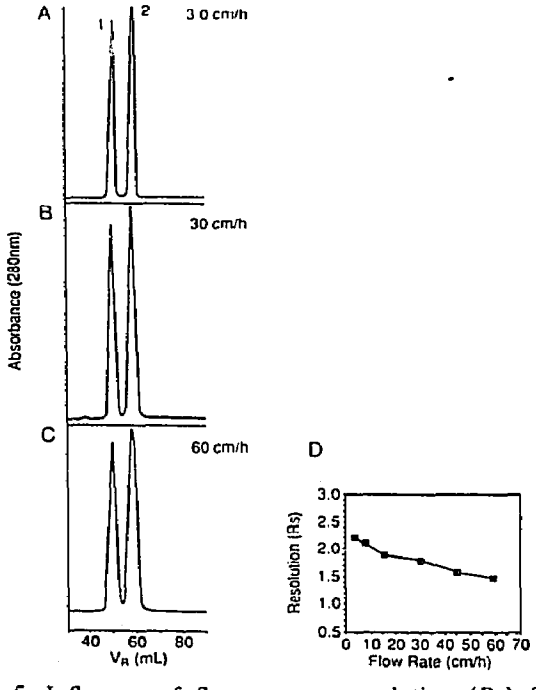


Fig. 5. Influence of flow-rate on resolution ($R_S$) for the cytochrome c(1)/rTAP(2) pair. Feed concentration was 0.75 mg/ml of each protein and injection volume was 1.7% $V_c$ (2.0 ml). Detection was by absorbance at 280 nm. Other conditions as in Table 1.

Several proteins exhibited $K_D$ values that did not correspond to their $M_r$. Lysozyme and poly-L-lysine eluted at volumes corresponding to $M_r$ values of 6300 and 2400, respectively. Retardation of lysozyme (p*I* 11.0) has been reported for Superdex 75 and other gel permeation media [13,14]. Several explanations have been proposed: (A) an "end-on-insertion" mechanism [15] resulting from the molecule's prolate ellipsoidal conformation, (B) ionic interactions between the positively-charged molecule and residual anionic groups on the gel matrix, (C) specific binding of the enzyme to matrix carbohydrates resembling its substrate, and (D) hydrophobic interactions. Ionic interactions are unlikely considering that the mobile phase contained 0.15 *M* NaCl, a concentration shown previously to reduce these with Sepharose, Sephadex and Superdex 75 media [14,16]. Furthermore, basic proteins such as cytochrome c (p*I* 10.2) and $\alpha$-chymotrypsinogen A (p*I* 8.8) showed good correlation between $K_D$ and $M_r$ values. The homopolymer poly-L-lysine has been shown to exist as a random coil in aqueous solution at neutral pH [17] and molecules in this conformation tend to elute earlier from a gel-permeation matrix than a globular protein of similar $M_r$ because their hydrodynamic radius is greater than that of a sphere. It is likely that the sample of poly-L-lysine is to some extent polydisperse with respect to its $M_r$. Consequently the $M_r$ is a mean $M_r$ and it is difficult to relate its $M_r$ to monodisperse molecules such as proteins.

Agarose–dextran based media may carry a residual negative charge resulting in retardation of proteins with a net positive charge at a given pH by electrostatic interactions [14,18]. In order

to determine if such effects were responsible for the aberrant migration of some of our test proteins, chromatography was performed with a mobile phase of 6.25 m*M* sodium phosphate, pH 7.2, 0.5 *M* NaCl. The $K_D$ values of lysozyme and poly-L-lysine were unchanged under these conditions, suggesting that their retardation is not primarily the result of ionic interaction with the matrix.

rHIR and rLAPP exhibited $K_D$ values approximately twice those expected based on $M_r$ values, which suggested that they might be dimeric structures.

Molecular mass determinations of rHIR by several methods, including gel permeation by HPLC [19] and SDS-PAGE under reducing conditions [20] have yielded values ranging from 9000 to 16 000. Only after reduction and alkylation followed by electrophoresis under denaturing and reducing conditions was an $M_r$ obtained that was consistent with that predicted from the cDNA. Mass spectrometric analysis of rHIR [21] and rLAPP [E. D. Lehman, unpublished data] have shown that both exist as monomers. The structures of rHIR have been determined by NMR [22] and the crystallographic structure of rHIR-thrombin complex has been solved at 2.3 Å resolution [23]. Both analyses show that rHIR possesses a compact $NH_2$-terminal head and a long polypeptide COOH-terminal tail. Such a protein would have a greater hydrodynamic radius than a globular protein of the same $M_r$, and would therefore be expected to have a smaller $K_D$. The data for rLAPP may also suggest that it is not a globular protein.

Synthetic *S. cerevisiae* $\alpha$1-mating factor was significantly retarded, exhibiting a $K_D$ of 2.7. This 13 residue peptide of $M_r$ 1684 contains 40% hydrophobic residues, including 3 aromatic residues. The possibility that hydrophobic interaction with aromatic residues was responsible for the retardation was suggested when L-tryptophan was chromatographed and exhibited a $K_D$ of 1.6. Interactions between aromatic amino acids have been shown previously for Sephadex media [24,25] as well as for TSK-Gel SW type media [26]. Proteins and polymers containing these residues may behave similarly. Lysozyme and chymotrypsinogen A display hydrophobic interactions with Superdex 75 at low pH (4.2) in the presence of 0.4 *M* NaCl [14].

The effect of hydrophobic interactions on $K_D$ was probed by adding organic solvent to the mobile phase. Chromatography was performed in a mobile phase consisting of 24% $CH_3CN$, 6.25 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. The $K_D$ of $\alpha$1-mating factor was reduced to 0.69, suggesting that hydrophobic interactions played a significant role in the retardation of this peptide. Similarly, the $K_D$ of L-tryptophan was reduced to 1.3. However, this mobile phase seemed to affect the porosity of the matrix. Polypeptides such as r-LAPP, r-TAP, and bombesin co-eluted with no linear resolution.

To determine whether electrostatic interactions between the $\alpha$-amino group of tryptophan and the matrix were causing the retardation, the analog compounds N-$\alpha$-methyl tryptophan and 3-indolepropionic acid were chromatographed, yielding $K_D$ values of 1.3 and 1.2, respectively. At pH 2 the $K_D$ of L-tryptophan increased to 1.4. The effect of salts on hydrophobic interactions with highly crosslinked hydrophobic macroreticular gels has been reported [24,25,27]. Sodium chloride at concentrations up to 1.0 *M* has little effect on either increasing or reducing the $K_D$ of tryptophan on Sephadex gels [25]. L-Phenylalanine, L-tyrosine and L-tryptophan were chromatographed yielding $K_D$ values of 1.0, 1.1 and 1.4, respectively. Injection of a mixture of the three amino acids confirmed that these values were significantly different and reproducible. Based on $M_r$ alone, the three should elute in the total volume of the column without any resolution. If significant hydrophobic interactions were occurring, tyrosine might be expected to elute prior to phenylalanine. Hydrogen bonding interactions with the gel could also occur.

### 3.2. Gel filtration of reduced and alkylated proteins in the presence of the strong denaturing agent guanidine hydrochloride

This method frequently is used to determine the subunit $M_r$ of proteins and peptides by gel

filtration by eliminating conformational effects [28]. Under such conditions, all species are assumed to exist in random coils. The behaviour of polypeptides under these condition was examined on Superdex 30 prep grade medium. Table 2 lists those used and their $M_r$ values after alkylation of thiols with 4-vinylpyridine. Fig. 6 shows the relationship between $K_D$ and $\log_{10} M_r$ in 6.25 m*M* sodium phosphate, pH 7.2, 0.15 *M* NaCl, 6 *M* guanidine hydrochloride. The results show that the $M_r$ exclusion was reduced to 10 000 and the resolving range extended to 85. The exclusion limit for proteins on Superdex 30 was effectively lowered upon their transition to the random coil conformation. Also, the presence of the chaotrope guanidine hydrochloride significantly reduced the hydrophobic interactions of tryptophan in accordance with its position in the Hofmeister series of chaotropes [25]. In a high-performance liquid chromatographic analysis comparing size exclusion of globular proteins and glycosaminoglycan (GAG) chains, the upper exclusion limit for GAGs was considerably lower on a given column than for globular proteins [29]. However, below approximately 1000 $M_r$, both types of molecules exhibited similar retention volumes. It was postulated that this is because low-molecular-mass peptides exist primarily as random coils, as do the high-molecular-mass GAGs.

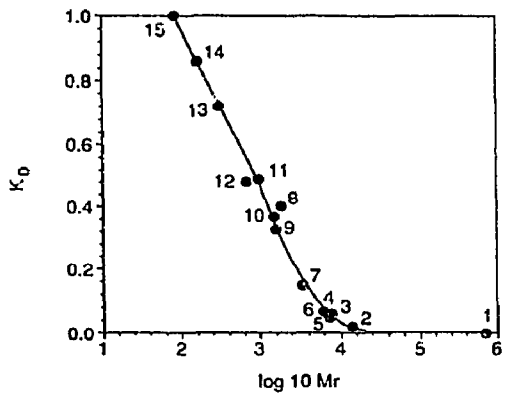


Fig. 6. $K_D$ of polypeptides *vs.* $\log_{10} M_r$ under denaturing conditions. Thyroglobulin-4VP was used to determine $V_0$ and 1% $NaNO_3$ was used to determine $V_T$. Sample numbers and experimental conditions are given in Table 2

Table 2
Data for proteins and peptides fractionated on Superdex 30 under denaturing conditions

| No. | Sample name | Molecular mass[a] | Recovery[b] (%) |
|---|---|---|---|
| 1 | Thyroglobulin-4VP | 695 000 | 95 |
| 2 | r-LAPP-4VP | 14 272 | 99 |
| 3 | r-TAPY1W/D10R-4VP | 7671 | 92 |
| 4 | r-TAP-4VP | 7607 | 99 |
| 5 | r-Hirudin-4VP | 7522 | 85 |
| 6 | r-echistatin-4VP | 6253 | 84 |
| 7 | Insulin B Chain-2($SO_3$) | 3496 | 95 |
| 8 | $\alpha_1$-Mating Factor | 1918 | 88 |
| 9 | Bombesin | 1620 | 94 |
| 10 | Substance P | 1552 | 99 |
| 11 | Oxytocin | 1007 | 25 |
| 12 | Pepstatin A | 686 | N.D.[c] |
| 13 | Glutathione | 307 | N.D. |
| 14 | L-Phenylalanine | 165 | N.D. |
| 15 | $NaNO_3$ | 85 | N.D. |

Polypeptides containing disulfide bonds (except oxytocin) were reduced and alkylated as described in Experimental. Chromatography was performed using a mobile phase of 6.25 m*M* sodium phosphate, 0.15 *M* NaCl, 6 *M* guanidine hydrochloride, pH 7.2. Flow-rate was 15 cm/h. Injection volume was 0.8% $V_c$ (1.0 ml). Detection was by absorbance at 280 nm or 220 nm.

[a] Calculated based on addition of one S-β-4-pyridylethyl group (FW 105) per mole sulfhydryl for the alkylated proteins.

[b] Determined as in Table 1.

[c] N.D.: not determined.

Chromatography in guanidine hydrochloride of the disulfide-containing proteins r-HIR and r-LAPP following reduction and alkylation results in better recoveries (Table 2) than were observed under native conditions. Substance P and $\alpha_1$-mating factor, which contain no disulfide bonds, also gave better recoveries under denaturing conditions. However, oxytocin, which contains one disulfide bond, was not reduced and alkylated and its poor recovery under native conditions (54%) was reduced to 25%. These results suggest that reduction and alkylation of disulfide bonds may be desirable in order to maximize recovery in the presence of guanidine hydrochloride.

### 3.3. Chromatographic resolution of recombinant polypeptides with Superdex 30 prep grade

Purified rTAP and synthetic echistatin showed good correlation with predicted $M_r$ values when chromatographed in aqueous mobile phase under non-denaturing conditions (see Fig. 1). However, a mutant form of rTAP (TAP Y1W/D10R) was also expressed and purified. Chromatographic analysis on Superdex 30 in non-denaturing aqueous mobile phase (Fig. 7A) showed that the mutant eluted later than the wild type polypeptide, although the former is larger by 64 mass units. The mutant has the potential for increased electrostatic and hydrophobic interactions with the matrix because of the arginine and tryptophan substitutions. The calculated net charges of the mutant and wild type proteins at pH 7 are −2.1 and −4.1, respectively. Chromatographing the polypeptides in the presence of 24% acetonitrile reverses the elution order (Fig. 7B). Addition of the organic solvent would be expected to decrease the hydrophobic contribution of the added tryptophan residue. The denaturing effect of the organic solvent might also lead to structural alterations of the molecule. r-TAP contains three disulfide bonds in a polypeptide with a $M_r$ of approximately 7000. The structure is believed to be fairly compact. Furthermore, in the course of purification the molecule is exposed to 70% acetonitrile–0.1% trifluoroacetic acid and retains its full biological activity [7]. The reduced and alkylated polypeptides co-migrated in the presence of guanidine hydrochloride (Fig. 7C); however, they eluted near the void volume of the column so the system is not suited for separating molecules in this $M_r$ range.

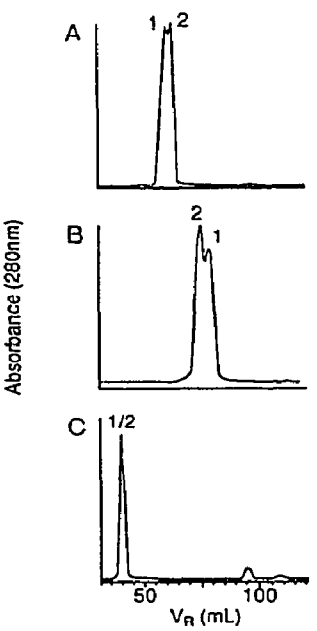


Fig. 7. Chromatography of a rTAP (1)/rTAP (Y1W/D10R) (2) mixture under various buffer conditions. (A) Mobile phase was 6.25 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.2. Flow-rate was 30 cm/h. Injection volume was 1.0 ml of a 0.25 mg/ml mixture. (B) Mobile phase was 24% $CH_3CN$, 6.25 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Flow-rate was 30 cm/h. Injection volume was 1.0 ml of a 0.5 mg/ml mixture. (C) Mobile phase was 6.25 m*M* sodium phosphate, 0.15 *M* NaCl, 6 *M* guanidine hydrochloride, pH 7.2. Flow-rate was 15 cm/h. Injection volume was 1.0 ml of a 0.25 mg/ml mixture.

One proposed application for Superdex 30 prep grade medium is for the separation of monomeric polypeptides in the 4000–10 000 $M_r$ range from oligomeric forms. The utility of Superdex 30 for this task was investigated using purified recombinant proteins. The separation of rTAP and cytochrome c (Fig. 4 D-F) illustrated how proteins with an $M_r$ difference factor of <2.0 can be separated. Depending upon the resolution required, concentrated solutions at fairly high load volumes can be resolved successfully. The relatively gradual loss of resolution in preparative separations with increasing flow-rates above 30 cm/h enables preparative separations to be carried out in 1–2 h.

## 4. Conclusions

Superdex 30 prep grade possesses the same characteristics which make Superdex 75 and Superdex 200 useful for preparative separations, namely a high degree of selectivity coupled with mechanical strength. The lower $M_r$ fractionation range offered by this medium should prove

useful to investigators working with small recombinant proteins and peptides. In our experiments, fractionation was obtained from 43 000 to 1000 $M_r$ for polypeptides. The linear fractionation range was found to be 24 000–3000 $M_r$. Resolution and process time appear to be slightly better than for the Superdex 75 and 200 media. Electrostatic, hydrophobic and hydrogen-bonding interactions are typical of those observed with similar macroreticular media and, within limits, can be compensated for by modification of the mobile phase.

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