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Estimation of peptide/protein molecular weights by high-performance molecular-sieve chromatography on agarose columns in 6 M guanidine hydrochloride*

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Abstract: Calibration curves showing a linear relationship between -log K_D and (molecular weight)$^{2/3}$ are presented for peptides/proteins subjected to molecular-sieve chromatography in 6 M guanidine hydrochloride on crosslinked gels of different agarose concentrations (9, 12 and 20%). These gels permit separation and estimation of molecular weights of peptides and proteins in the range 1000 and 90 000 daltons. Surprisingly, the exclusion limits in the presence of guanidine are only slightly affected by the gel concentration. Another unexpected result was that the exclusion limit in 6 M guanidine–HCl increased after crosslinking. In the absence of this agent a crosslinked gel has the same exclusion limit as the non-crosslinked one. Separations of model proteins are shown.

Keywords: *HPLC; molecular-sieve chromatography; size exclusion chromatography; protein; guanidine hydrochloride; molecular weight determination; agarose.*

Introduction

Molecular-sieve chromatography in 6 M guanidine hydrochloride [1, 2] has been widely used as a method for estimation of molecular weights and chain lengths of peptides and proteins. In 6 M guanidine hydrochloride, reduced and alkylated peptide/protein chains behave as randomly coiled linear homopolymers [3]. Molecular-sieve chromatography in this solvent of peptides/proteins, thus treated, therefore affords a satisfactory estimation of molecular weights and consequently of peptide/protein chain lengths (number of amino acids) [1, 2]. Even the molecular weights of most glycoproteins (if the carbohydrate content is not excessively high) can be well estimated by molecular-sieve chromatography in 6 M guanidine hydrochloride [4, 5]. Recently, molecular-sieve chromatography in the HPLC mode in this medium has been introduced [6, 7], e.g. using the

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TSK-GEL SW columns. These silica-based rigid gels have the advantage over the classical, soft gels, that they permit higher flow rates (lower run times), but have the drawback of a lower V_i/V_o ratio (pore volume over void volume) [8]. However, one of the classical gel materials, agarose, has at high concentrations, particularly after crosslinking, the mechanical stability required for an HPLC packing material, as well as a high V_i/V_o ratio [9–11].

This paper deals with the determination of molecular weights of peptide/protein chains on such gels (agarose concentration: 9–20%) in the presence of guanidine hydrochloride. The separation potential of the agarose columns is illustrated with model proteins and peptides.

Instrumentation and Materials

The pump used was an LKB 2150 HPLC pump with an LKB 2152 HPLC controller; the UV detector an LKB 2158 Uvicord SD; the recorder/integrator an LKB 2220 Recording Integrator (LKB, Bromma, Sweden). The samples were applied by a loop valve injector (Rheodyne, Berkeley, CA).

The peptides and proteins used are listed in Table 1. The agarose was from Réactifs IBF (Villeneuve la Garenne, France); divinyl sulfone (DVS) and guanidine hydrochloride (purum) from Fluka AG (Buchs, Switzerland); dithiothreitol (DTT) and iodacetamide from Sigma (St Louis, MO); 2-mercapto-ethanol from Roth (Karlsruhe, FRG); and Blue Dextran (molecular weight 2 000 000) from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Table 1
Peptides and proteins used for the calibration curves shown in Fig. 1

Peptide/protein	Molecular weight	$(Mw)^{2/3}$	Carbohydrate (%)	Source
Bradykinin potentiating factor	1100	107	—	1
Neurotensin	1670	141	—	1
Insulin (bovine), A chain	2300	174	—	2
B chain	3300	222	—	2
Insulin, B chain (di-S-sulfonate, Na-salt)	3300	222	—	1
ACTH (porcine)	4500	273	—	1
Notechis III:4 (a neurotoxin from the Australian tiger snake)	8050	402	—	3
Cytochrome C (horse)	12 400	536	—	4
Ribonuclease A (bovine)	13 700	573	—	5
Myoglobin (horse)	17 200	666	—	4
β-lactoglobulin (bovine)	18 300	649	—	4
γ-globulin, L-chain (human)	23 000	809	—	6
Chymotrypsinogen A (bovine)	25 000	855	—	5
Ovalbumin (hen)	43 000	1227	3.2	5
Enolase (bakers yeast)	44 000	1246	—	7
γ-globulin, H-chain (human)	51 500	1384	4.9	6
Serum albumin (human)	66 400	1640	—	6
Transferrin (human)	80 000	1857	5.9	6

Sources: 1, Serva (Heidelberg, FRG); 2, Sigma (St Louis, MO); 3, Gift from Dr D. Eaker; 4, Bio Rad (Richmond, CA); 5, Pharmacia Fine Chemicals (Uppsala, Sweden); 6, Kabi (Stockholm, Sweden); 7, Gift from Dr G. Petterson.

Experimental

The agarose beads were prepared, crosslinked, and packed as previously described [10]. The 6 M guanidine hydrochloride solution was purified according to Fohlman *et al.* [12], and buffered with 0.1 M sodium phosphate buffer, pH 6.5. Reduction and alkylation was performed in 6 M guanidine hydrochloride buffered with 0.1 M Tris–HCl, pH 8.6, containing 2 mM EDTA. A ten-fold excess of DTT was added to the peptide/protein solutions which had a concentration of about 1.5 mg ml^{-1}. The mixture was kept under nitrogen at room temperature for 4 h (overnight in the case of albumin). Proteins thus reduced were then alkylated, under nitrogen, for 2 h at room temperature with iodoacetamide in a quantity equivalent to the total free thiol groups. Mercaptoethanol (10 μl) was added. The amount of peptide/protein injected per run varied from 5 to 50 μg. The flow rate was 0.1 ml min^{-1}. The peptide/protein distribution was determined by continuous absorption measurements at 280 nm. The equation $K_D = (V_e - V_o)/(V_T - V_o)$ was employed for the estimation of the distribution coefficient K_D, where V_e is the elution volume, V_o the void volume (determined with Blue Dextran) and V_T the elution volume for a small molecule, such as deuterium oxide or chromate [10]. The function -log K_D was plotted against $(Mw)^{2/3}$ according to the theory of Hjertén [13] based on a thermodynamic treatment of molecular-sieve chromatography (Fig. 1).

Results and Discussion

Non-crosslinked gels have not been used in this study since they give relatively low flow rates in the highly viscous 6 M guanidine hydrochloride. Figure 1 shows the calibration curves for 9, 12 and 20% agarose, crosslinked with DVS. The linear relationship observed between -log K_D and $(Mw)^{2/3}$ indicates that all of the proteins have the same type of conformation, as expected [3]. A surprising finding is that the separation range, i.e. the molecular weight range within which proteins/peptides can be separated, is almost independent of the agarose concentration used (9, 12 and 20%) and encompasses peptides and proteins in the approximate size range 1000–80 000 daltons (10–700 amino acids). Interestingly, an agarose concentration as low as 6% gives a similar separation range (from 3000 to 80 000 daltons as reported by Ansari and Mage [14]). In other words, 6–20% agarose gels have a comparable pore size in the presence of guanidine hydrochloride, but are very different in the absence of this denaturing agent [10]. The reason for this has not been explored, but may be attributable to disruption of hydrogen bonds between polymer chains (guanidine hydrochloride is known to break such bonds).

The slopes of the calibration curves for proteins/peptides on 9, 12 and 20% agarose were 0.014, 0.012 and 0.015, respectively, indicating that the slope is independent of gel concentration. This unexpected finding does not apply for either the non-crosslinked or the crosslinked agarose gels in the absence of guanidine hydrochloride (cf. [10], where the same 9, 12 and 20% agarose beads were used as in this series of experiments). It is therefore likely that the guanidine hydrochloride affected the 9, 12 and 20% crosslinked agarose gels differently because of some differences in chemical structure.

The gels might thus differ with regard to: (1) the chain lengths of the agarose molecules in the 9, 12 and 20% gel beads, which were prepared from different batches of agarose; or (2) the degree of crosslinking obtained for these two gels. These two parameters may be of some importance for the effective pore size of an agarose gel, since

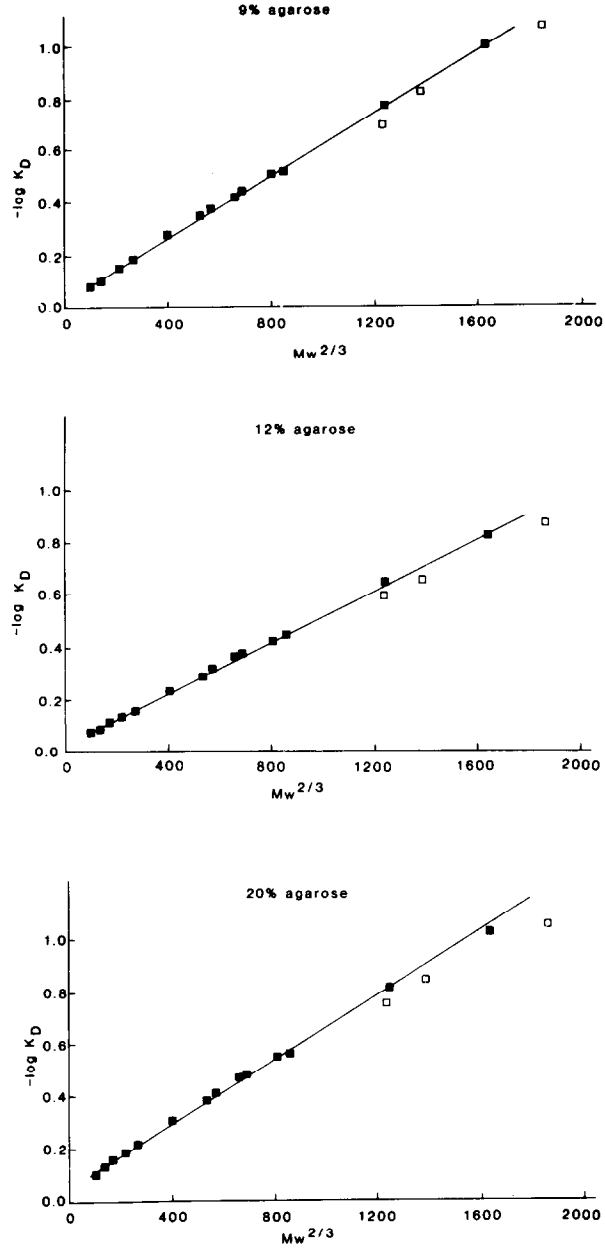


Figure 1
Calibration curves for 9, 12 and 20% agarose in 6 M guanidine hydrochloride, $-\log K_D$ was plotted versus $(Mw)^{2/3}$, for carbohydrate-free peptides/proteins ■ and glycoproteins □.

agarose chains are kept together in bundles by hydrogen bonds and these are known to be affected by guanidine hydrochloride. That the degree of crosslinking has an influence on the pore size is evident from our finding that crosslinking of a 20% agarose gel increased the exclusion limit for proteins from 60 000 to 90 000 daltons in the presence of guanidine hydrochloride. In the absence of this agent, a crosslinked gel has the same exclusion limit as the non-crosslinked one.

From the plot in Fig. 1 one can conclude that points corresponding to glycoproteins fall only slightly below the straight line obtained for carbohydrate-free proteins, which means that only a small error is introduced when the molecular weights of glycoproteins with a moderate carbohydrate content (up to 6% in this study) are estimated with the aid of standard non-conjugated proteins [4, 5]. A similar error is obtained upon electrophoresis of glycoproteins in polyacrylamide gels in the presence of SDS (SDS–PAGE) [15].

The difficulties in removing the last traces of UV-absorbing impurities in guanidine hydrochloride solutions make it very difficult to detect proteins and peptides at 210–220 nm, where the peptide bond has very strong absorption. Therefore, peptides lacking tryptophan and tyrosine residues cannot easily be detected by UV-measurements in the presence of 6 M guanidine hydrochloride.

A chromatographic estimation of molecular weights of peptides and proteins as described in the present work takes 50–100 min, depending on the flow rates used. This should be compared with the much longer time required for SDS–PAGE and for molecular sieving on classical soft gel materials. The high V_i/V_o ratio of agarose gels (2.5, 2.3 and 2.0 for the 9, 12 and 20% gels, respectively, under the conditions used in this study) contributes to the high resolution of agarose gels. Figure 2 gives a visual picture of the resolution and shows, unexpectedly, that the operational separation range extends down to compounds of very low molecular weight. Using the calibration curve in Fig. 1

Figure 2
Molecular-sieve chromatography of model peptides/proteins in the presence of 6 M guanidine hydrochloride. The sample, containing Blue Dextran (a), transferrin (b), ovalbumin (c), chymotrypsinogen A (d), ribonuclease A (e), notechis III:4 (f), ACTH (g) and neurotensin (h) (see Table 1 for molecular weights) was chromatographed on a column packed with 20% agarose beads with diameters varying from 5 to 15 μm. Sample amount: about 5 μg of each component in a total volume of 20 μl. Bed dimensions: 0.6 × 34.5 cm. Flow rate: 0.1 ml min $^{-1}$. The last peak (i) corresponds to some reaction product(s) from the reduction/alkylation of the sample. The resolution was slightly less when the flow rate was increased to 0.2 ml min^{-1}, which corresponded to a run time of 50 min.

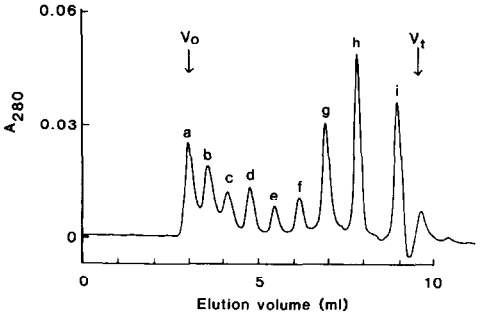
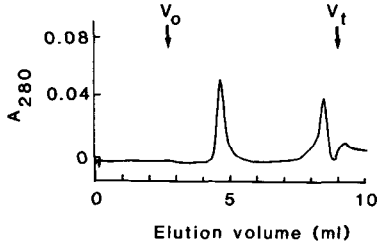


Figure 3
Molecular weight estimation of human growth hormone. About 10 μg, in a volume of 50 μl, was chromatographed on the same column and under conditions similar to those used in the experiment shown in Fig. 2.



for the 20% agarose column the molecular weight of human growth hormone, prepared according to Roos *et al.* [16], was estimated as 21 800 (cf. chromatogram in Fig. 3). This value agrees very well with the value 21 500 obtained from the known amino acid sequence [17].

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