Anomalous Molecular Weights of Proteases in Gel Chromatography

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SUMMARY. Attention is drawn to the fact that, in comparison to those values derived from sedimentation equilibrium experiments, gel chromatographic methods give anomalously low molecular weights for a variety of proteases. This appears to be particularly true for extracellular proteases of broad specificity produced by bacteria and fungi.

Of the various methods available for the determination of the molecular weight of proteins, sedimentation equilibrium, SDS gel electrophoresis and gel chromatography are the most popular. Of these three techniques, sedimentation equilibrium alone offers a direct approach to the experimental problem. The other two methods require the use of protein standards of known molecular weight (MW) and therefore the assumption that the unknown protein will behave similarly to the standards. Linear relationships have been established in SDS gel electrophoresis for R_f versus log MW,[1] and in gel chromatography for V_e versus log MW[2] for a wide variety of proteins well characterised with respect to their MW.

Using these three techniques we have examined the MW of the thermostable, extracellular protease of the fungus *Malbranchea pulchella* var. *sulfurea* ("Thermomycolase").[3] The sedimentation equilibrium experiments performed under a variety of conditions all indicate a MW of 32,000 for the DFP inhibited enzyme.[3] This value is confirmed by SDS-gel electrophoresis (33,000) when the proper sample preparation

technique is used (Ref. 4. Method 1). However, in gel chromatography we consistently find elution volumes that correspond to much lower MW's (11,000-17,000). We used a standard Pharmacia K25/45 column with flow adapters, packed with Sephadex G100 superfine (Lot No. 4078, Pharmacia Fine Chemicals) and equilibrated with 0.1 M glycine-NaOH buffer (pH = 8.0) containing 0.1 M NaCl and various concentrations of $CaCl_2$. The experiments were carried out at 4°C and a flow rate maintained at 0.2 ml/min (0.04 ml/cm^2 min) by a LKB Varioperpex peristaltic pump. Fractions of 1.2 ml were collected and elution volumes were estimated from the weight of the tube contents. The elution volume of Blue Dextran (V_0) was reproducible to 1% under these conditions. The calibrated column showed the expected linear relationship between K_{AV} and log MW[5] for a variety of standard proteins (Fig. 1). K_{AV} values for Thermomycolase varied from 0.45 to 0.60 (Table 1). The elution position of active Thermomycolase was established from activity measurements[3] and when possible OD_{280} values; that of DIP-Thermomycolase by OD_{280} only.[3] Active Thermomycolase underwent autolysis during the experiments resulting in a low recovery of protein activity. We know that this autolysis is

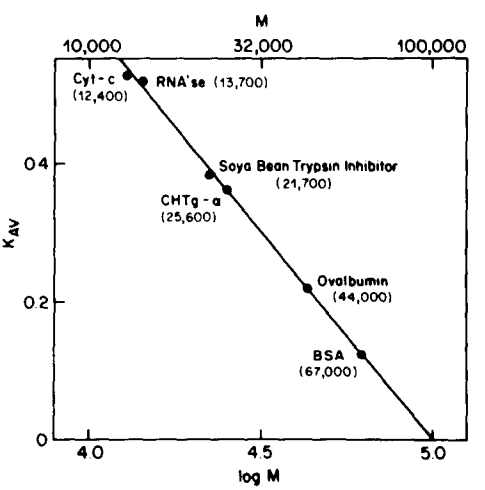

Fig. 1. Calibration Plot for the Sephadex G100 Column

Ca^{+2} dependent[3] being especially prominent at $[Ca^{+2}] < 10^{-4}$ M. Inspection of the results in Table 1, obtained with 0.1 mg/ml

Table 1: Elution Volumes and Corresponding MW's of Thermomycolase in Sephadex G-100 Gel Chromatography

Sample	Concn.* (mg/ml)	Concn. $CaCl_2$ (M)	K_{AV}	MW
DIP-Protease	10.0	0**	0.601	11,200
Active Protease	0.1	10^{-4}	0.504	13,600
Active Protease	0.1	10^{-3}	0.469	15,800
Active Protease	0.1	10^{-2}	0.486	14,800
Active Protease	0.1	10^{-1}	0.469	15,800
Active Protease	14.5	10^{-2}	0.451	17,000
DIP-Protease	15.0	10^{-2}	0.450	17,000

* original concentration of the sample (1 ml) applied to the column.

** no $CaCl_2$ added to the buffer solution.

of active protease, suggests that the Ca^{+2} dependent autolysis might influence the elution volume; a relatively low MW being obtained in the presence of 10^{-4} M Ca^{+2}. However, we do not understand why DIP-Thermomycolase, which is not autolytic (95% recovery of MW 32,000[3]), elutes at volumes corresponding to similarly low MW values. The only conclusion that we can draw from our data, therefore, is that this protease behaves anomalously for reasons presently unknown.

We report these observations because it is clear that a number of other proteases also behave in this fashion (Table 2).

Table 2. MW's of Proteolytic Enzymes Determined by Various Techniques

	Organism	Name	Sedimentation Equilibrium	SDS Gel Electrophoresis	Gel Chromatography
)	*Malbranchea Pulchella*	Thermomycolase	32,000[3]	33,000[3]	11,000-17,000
)	*Bacillus Thermoproteolyticus*	Thermolysin	34,000[6]	37,000-38,000[7]	28,000[6]
)	*Bacillus Subtilis*	Neutral Protease	38,000-42,000[8]	---	26,700[8]
)	*Bacillus Subtilis*	Alkaline Protease	32,500[9]	---	21,000[9]-22,000
)	*Staphylococcus Aureus*	Protease II	---	---	12,500[10]
)	*Actinidia Chinensis*	Sulfhydryl Protease	---	---	12,800[11]
)	*Myxobacter*	AL-1 Protease	14,000[12]	---	8,700[13]

The sedimentation equilibrium results have been shown to agree very well with chemically determined MW in two cases [Table 2, (b) and (g)]. The two MW's determined by gel chromatographic techniques only [Table 2, (e) and (f)] are, in our view, suspect until further characterisation data is obtained by other techniques. In a few cases[10,13] there has been speculation that the basicity of the proteases might be implicated in a possible retardation on Sephadex. The use of a BioGel matrix[10] did not, however, give a significantly different result for the apparent MW. Further, since Thermomycolase is not a basic protein (pI = 6.0),[14] the anomalous behaviour observed in gel chromatography cannot be accounted for in terms of protein basicity alone and other explanations must be examined.

In the light of the above observations (Table 2) it would appear that caution should be exercised in the use of gel chromatographic methods alone for the characterisation of proteases with respect to their molecular weight.

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