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Are Molecular Weights of Proteins Determined by Superose 12 Column Chromatography Correct?

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Our research on several proteins indicates that accurate molecular weights cannot be determined by Superose 12 column chromatography. In support of this statement, we present data on molecular weights of purified red kidney bean α-amylase inhibitor (RKB αAI) and white kidney bean α-amylase inhibitor (WKB αAI) to document this problem. The molecular weight of purified RKB αAI determined by Sephadex G-100 gel filtration, polyacrylamide gel electrophoresis, Superose 12 gel filtration and cDNA were 49.0, 51.0, 22.9, and 49.805 kDa (not glycosylated), respectively. The molecular weights of WKB αAI by several methods were as follows: Sephadex G-100 gel filtration, 51.0 kDa; Superose 12 gel filtration in 0.2 M NaCl buffer, 23.1 kDa; polyacrylamide gel electrophoresis (PAGE), 51.0 kDa; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 45.0 kDa; multiangle laser light scattering (MALLS), 49.940 kDa; laser-assisted time-of-flight mass spectrometry (LATOFMS), 56.714 kDa; and cDNA sequence (with 12.2% carbohydrate), 55.9 kDa. The data indicate there is ionic interaction between proteins and the matrix of Superose 12 in low ionic strength buffers and hydrophobic interaction at higher ionic strength buffers. Researchers should be cautious when using Superose 12 columns for molecular weight determinations.

KEYWORDS: Protein molecular weight accuracy; column chromatography; ionic/hydrophobic interaction with column matrixes; protein structure

INTRODUCTION

In protein and enzyme purification and characterization, it is essential to determine their molecular weights. Sephadex gel filtration is one of the prevalent methods used in molecular weight determination (*1*). However, the Superose 12 gel filtration method is now used more frequently for molecular weight determinations, using the FPLC (Fast Protein Liquid Chromatography) system. The Superose 12 matrix is a cross-linked agarose (in contrast to cross-linked dextran for Sephadex gels). Both matrixes separate proteins on the basis of molecular weight (presumably).

In our laboratory, the determined molecular weight of red kidney bean α-amylase inhibitor (RKB αAI) using a calibrated Superose 12 column was 22.9 kDa (running buffer: 0.2 M sodium acetate/acetic acid, 0.15 M NaCl, 0.02% NaN_3, pH 5.2) (*2*). However, the molecular weight of RKB αAI was previously reported to be 49 kDa based on a Sephadex G-100 gel filtration method (*3*). Earlier, the elution volumes of lipoprotein fractions on Superose 6 columns were reported to be sensitive to the mobile phase pH and ionic strength (*4*). In this paper, RKB αAI and WKB αAI (both purified to homogeneity in our lab) were used to compare molecular weights using the Superose 12 column chromotography in relation to molecular weights by Sephadex G-100 column chromatography, and by polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by multiangle laser light scattering (MALLS), laser-assisted time-of-flight mass spectrometry (LATOFMS), and by cDNA sequence. Horse heart cytochrome *c*, Kunitz soybean trypsin inhibitor, chicken egg white ovalbumin, bovine serum albumin and human transferrin from Sigma Chemical Co. were used to standardize the columns.

MATERIALS AND METHODS

Red kidney beans (bin 6110) were obtained from a Davis, California grocery store. White kidney beans and black beans were harvested from the University of California farm. Porcine pancreatic α-amylase (type 1-A, DFP treated, 2x crystallized), DEAE-cellulose, and CM-cellulose were from Sigma Chemical Co., St. Louis, MO. Sephadex G-100 and Superose 12 were from Pharmacia LKB Biotechnology, Pleasant Hill, CA. The five standard proteins, horse heart cytochrome *c* (MW 12.384 kDa), Kunitz soybean trypsin inhibitor (MW 20.0 kDa), chicken egg white ovalbumin (MW 45.0 kDa), bovine serum albumin (MW 65.0 kDa) and human transferrin (MW 80.0 kDa) were pure proteins from Sigma Chemical Co. All other reagents were reagent grade.

Purification of Inhibitor. The RKB αAI was purified to homogeneity, as described by Powers and Whitaker (*3*), but some steps were modified as needed. These included addition of a Sephadex G-100 gel filtration chromatography step (fractionation range of 4.00−150.0 kDa). The linear gradients of NaCl concentration used to elute the proteins

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Table 1. Purification of Red Kidney Bean α-Amylase Inhibitor (RKB αAI)

step	vol (mL)	activity (units/mL)	total activity (units × 1000)	protein (mg/mL)	specific activity (units/mg)	recovery activity (%)	fold purification
1. extract	370	4980	1840	3.95	1260	100.0	1.0
2. heat treatment	317	4820	1400	2.54	1740	76.1	1.4
3. ethanol fracitonation	138	9490	1310	2.50	3800	71.1	3.0
4. DEAE-cellulose	433	1850	800	0.389	4750	43.4	3.8
5. CM-cellulose	66	11800	780	0.967	12200	42.4	9.7
6. Sephadex G-100	16	13100	210	0.923	14200	11.4	11.3

Table 2. Effect of Ionic Strength on V_n/V_o of Purified RKB αAI and Standard Proteins Chromatographed Separately on a Superose 12 Column

standard proteins	MW (kDa)[a]	V_n/V_o[b] 0 M[c]	0.05 M[c]	0.2 M[c]	0.4 M[c]	1.0 M[c]
1. human transferrin	80.0	1.59	1.70	1.73	1.74	1.74
2. bovine serum albumin	66.0	1.59	1.66	1.76	1.75	1.75
3. chicken ovalbumin	45.0	1.70	1.80	1.85	1.87	1.87
4. Kunitz soybean trypsin inhibitor	20.1	1.83	1.95	2.02	2.05	2.11
5. cytochrome *c*	12.4	1.73	2.19	2.16	2.14	2.12
R^2		0.656[d]	0.939[d]	0.995[d]	0.993[d]	0.959[d]
RKB αAI (MW, kDa)[d]		14.0[e]	30.7[e]	22.9[e]	16.3[e]	*f*

[a] Literature values. [b] Relative retention time, where V_n is the elution volume for a given protein, and V_o is the elution volume for Blue Dextran 2 million. [c] Running buffer contained 0.02 M potassium phosphate, 0.02% sodium azide and various concentrations of NaCl at pH 7.6. [d] R^2 with different concentrations of sodium chloride for the standard curve in the specific buffer used. [e] The calculated MW for RKB αAI based on the standard curve in the specific buffer used. [f] MW is not available for this V_n/V_o since it was located outside the linear range of the standard curve. It was <14.0 kDa.

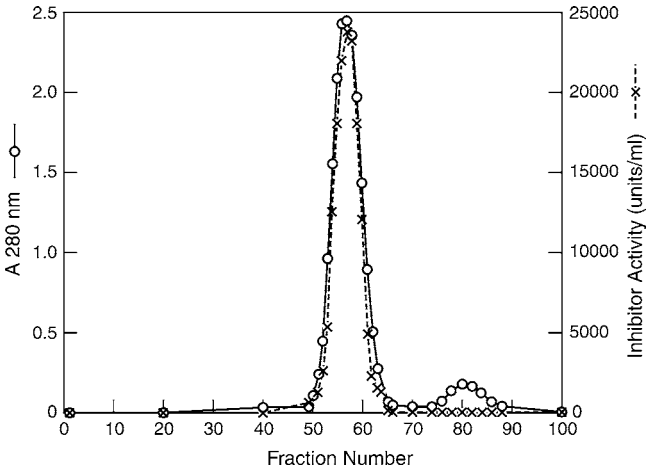

Figure 1. Chromatogram of purified red kidney bean α-amylase inhibitor (RKB αAI) using a Sephadex G-100 column. Column size: 2.54 × 65 cm. Elution buffer: 0.02 M potassium phosphate, 0.05 M NaCl, 0.02% NaN_3, pH 6.9. Sample size: 78.5 mg inhibitor from previous CM-cellulose column chromatography (see **Table 1**).

were modified to 0.01−0.22 M and 0−0.20 M for DEAE-cellulose and CM-cellulose column chromatography, respectively. See **Figure 1** and ref *3* for other details of methodology.

Inhibitory Activities. Inhibitory activities of RKB αAI and WKB αAI against procine pancreatic α-amylase (EC 3.2.1.1; type 1A, Sigma) were determined by the method of Bernfeld (*5*) with modification. The substrate was 1% $NaBH_4$-reduced starch (*6*) in 0.04 M potassium phosphate buffer, pH 6.9, containing 0.05 M NaCl. A mixture of porcine pancreatic α-amylase solution (1×10^{-7} M, 0.1 mL), and inhibitor solution (0.1 mL) was incubated in a water bath for exactly 30 min at 30 °C, pH 5.4, prior to adding substrate solution (0.8 mL of 1% $NaBH_4$-reduced starch). The enzyme−substrate reaction time was 5.0 min. A standard curve for determining products was prepared using maltose. One inhibitor unit is defined as that amount of inhibitor which gives 50% inhibition of an aliquot of porcine pancreatic α-amylase that releases reducing sugar equivalent to 0.1 mg of maltose per min at pH 6.9 and 30 °C when acting on 1% $NaBH_4$-reduced starch for 5 min.

Table 3. Molecular Weight of White Kidney Bean α-Amylase Inhibitor Determined by Several Methods

method	molecular weight (kDa)
gel filtration[a]	51.0
PAGE[b]	51.0
SDS-PAGE[c]	45.0
MALLS[d]	49.940
LATOFMS[e]	56.714
cDNA[f]	49.805
cDNA with 12.2% carbohydrate	55.9

[a] Method of Whitaker (*1*) on Sephadex G-100 column. [b] Polyacrylamide gel electrophoresis method of Hedrick and Smith (*9*). [c] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis method of Weber and Osborne (*10*). [d] Multiangle laser light scattering (MALLS) (*13*). [e] Laser-assisted time-of-flight mass spectrometry (LATOFMS) (*13*). [f] cDNA sequence, before posttranslational modification of the protein by 12.2% carbohydrate (*13*).

Protein Analysis. Protein concentration was determined by the Lowry method (*7*) using crystallized bovine serum albumin (Sigma) as a standard. Protein concentration in fractions from column chromatography was determined by absorbance at 280 or 214 nm also. The molar concentration of solutions of porcine pancreatic α-amylase was determined from absorbance at 280 nm based on $A_{1\%} = 24.0$ ($L \cdot g^{-1}$) and a MW of 52.1 kDa (*8*).

Molecular Weight Determination. The following purified proteins were used. Human transferrin, bovine serum albumin, ovalbumin, Kunitz soybean trypsin inhibitor, horse heart cytochrome *c* and Blue Dextran 2000 from Sigma were used to calibrate the Sephadex G-100 and Superose 12 columns, determine V_o, and check column packing.

Gel Filtration. Sephadex G-100 was used to determine the molecular weights of RKB αAI and WKB αAI based on the method of Whitaker (*1*). FPLC with a Superose 12 column was also used in molecular weight determination of RKB αAI.

Gel Electrophoresis. The WKB αAI molecular weight was determined also by the PAGE method of Hedrick and Smith (*9*) and the SDS-PAGE method of Weber and Osborne (*10*) (**Table 3**).

Other Methods Used. The molecular weight of WKB αAI was determined also by MALLS (*13*), by the LATOFMS (*13*), and by the cDNA sequence method (*13*) (see **Table 3**).

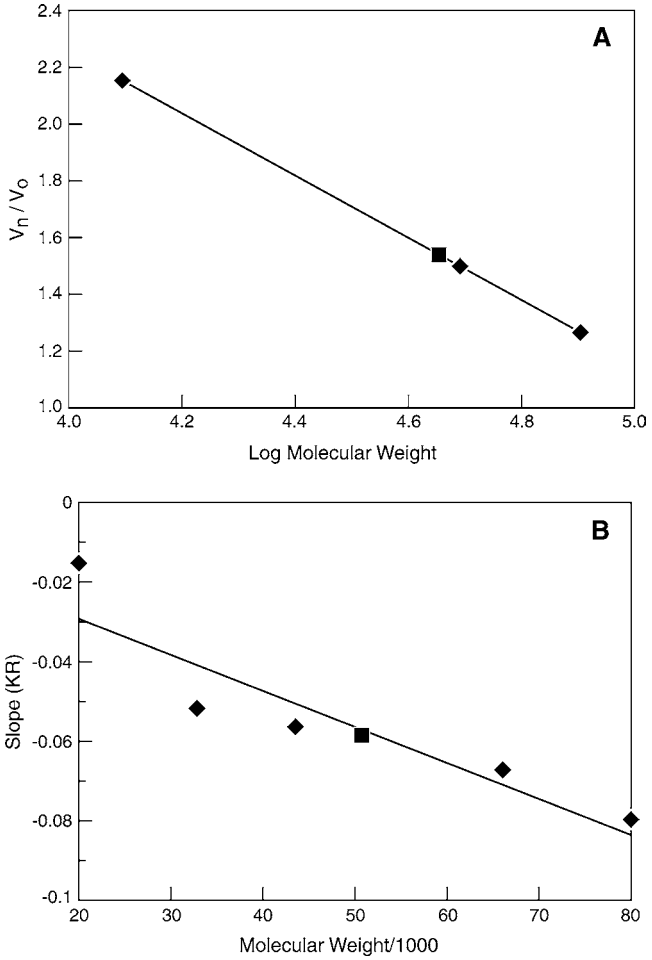

Figure 2. (**A**) Molecular weight determination of purified red kidney bean α-amylase inhibitor (RKB αAI; **Table 1**) using a standardized Sephadex G-100 column. The buffer contained 0.02 M potassium phosphate, 0.2 M NaCl, 0.02% NaN_3, at pH 6.9. Glass column size: 2.54 × 65 cm. The standard proteins used were (from left to right): 1, horse heart cytochrome *c*; 3, chicken ovalbumin; 4, BSA. The RKB αAI is No. 2 (■). (**B**) Molecular weight determination by gel electrophoresis for purified red kidney bean α-amylase inhibitor using 6, 8, 10, and 12% polyacrylamide gel concentrations (*9*). The standard proteins at 1 mg/mL were (from left to right): 1, horse heart cytochrome *c* (12.4 kDa); 2, Kunitz soybean trypsin inhibitor (20.1 kDa); 3, chicken ovalbumin (45.0 kDa); 5, BSA (66.0 kDa); 6, human transferrin (80.0 kDa). The RKB αAI is No. 4 (■).

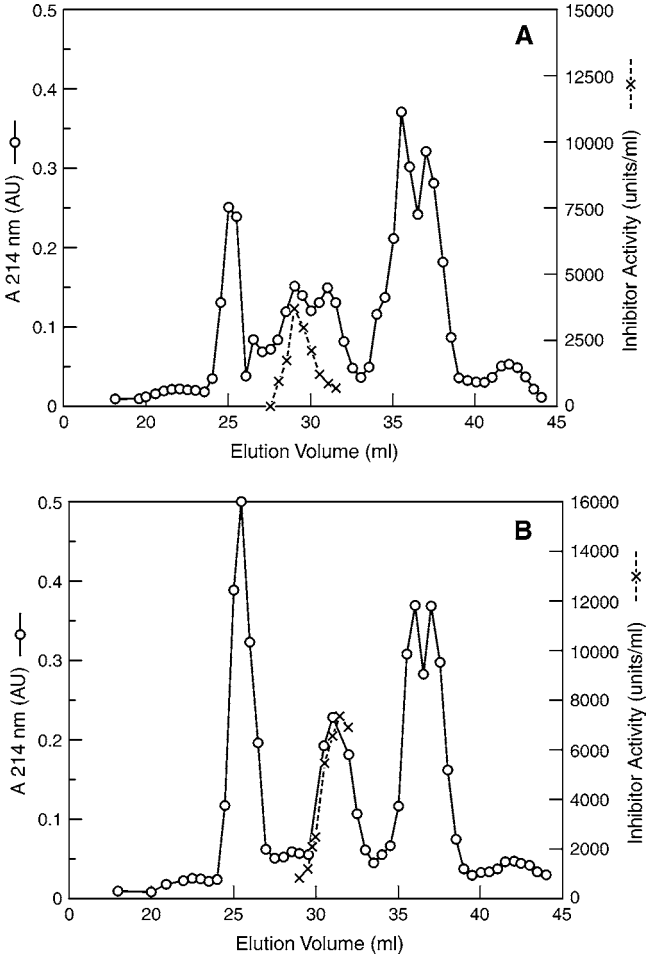

Figure 3. (**A**) Chromatogram of a crude extract of red kidney bean α-amylase inhibitor (RKB αAI) using Superose 12 column chromatography. Glass column size: 1 × 30 cm; elution buffer: 0.02 M sodium phosphate, 0.2 M NaCl, 0.02 NaN_3, pH 7.0. (**B**) Chromatogram of the same crude extract of RKB αAI using a Superose 12 column chromatography. Glass column size: 1 × 30 cm; elution buffer: 0.2 M sodium acetate/acetic acid, 0.15 M NaCl, 0.02% NaN_3, pH 5.2.

RESULTS AND DISCUSSION

Purification of Red Kidney Bean α-Amylase Inhibitor and White Kidney Bean α-Amylase Inhibitor. Purification of RKB αAI is summarized in **Table 1**. Heating of the aqueous extract for 20 min at 60 °C, pH 4.0, to denature some other proteins gave 1.4-fold increase in specific activity. Ethanol fractionation (40−80% cut; 0 °C) resulted in an additional 1.6-fold increase in specific activity. Successive chromatography on DEAE-cellulose, CM-cellulose and Sephadex G-100 columns (final step, **Figure 1**) resulted in 0.8, 5.9 and 1.6-fold further increases in specific activity. The total fold purification was 11.3. Very similiar results were obtained for WKB αAI (*13*).

Molecular Weights of Red Kidney Bean α-Amylase Inhibitor and White Kidney Bean α-Amylase Inhibitor. Sephadex gel filtration columns are commonly used in molecular weight (MW) determination for bean αAIs and other proteins (*3*, *10*−*14*). The gel electrophoresis method of Hedrick and Smith (*9*) is another commonly used method for MW determination. Therefore, we determined the MW of RKB αAI and WKB αAI by these two methods for comparison with the Superose 12 column chromatography method. In both chromatographic methods, the running buffer contained 0.02 M potassium phosphate, 0.02% sodium azide, and varied concentrations of NaCl, all at pH 7.6 (**Table 2**).

The results were quite different. The MW of purified RKB αAI determined by the Sephadex G-100 column method was 49 kDa (**Figure 2A**). This MW is identical to that reported by Powers and Whitaker (*3*) by the same method. The MW of RKB αAI by the gel electrophoresis method was 51 kDa (**Figure 2B**). The MW of WKB αAI was 51.0 kDa by both the Sephadex G-100 column and the gel electrophoresis (PAGE) method (**Table 3**). However, the MW of RKB αAI was determined to be 22.9 kDa by FPLC Superose 12 column chromatography (column size, 1 × 30 cm; running buffer contained 0.02 M potassium phosphate, 0.02% sodium azide and 0.2 M sodium chloride, at pH 7.6) (**Table 2**).

pH and Type of Buffer on MW of RKB αAI. Sodium phosphate buffer (0.02 M, 0.02% sodium azide and 0.2 M NaCl, pH 7.0) (**Figure 3A**) and sodium acetate/acetic acid buffer (0.02 M, 0.02% sodium azide and 0.2 M NaCl, pH 5.2) (**Figure 3B**) were used to determine the elution volume of RKB αAI (using inhibitor activity) in a crude bean extract by Superose 12 chromatography. Except for change of buffer, all other experi-

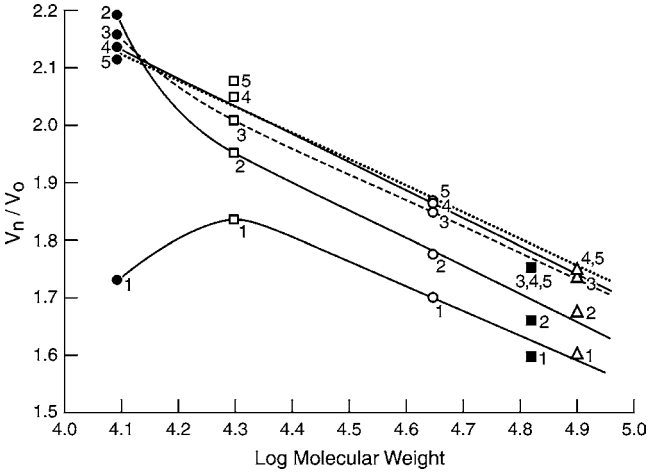

Figure 4. Plot of effect of sodium chloride concentration (ionic strength) on V_n/V_o of five standard proteins chromatographed on Superose 12 columns, based on data of **Table 2**. The standard proteins are ●, cytochrome *c*, 12.4 kDa; □, Kunitz soybean trypsin inhibitor, 20.1 kDa; ○, chicken egg white ovalbumin, 45.0 kDa; ■, bovine serum albumin, 66.0 kDa; and △, human transferrin, 80.0 kDa. The lines from bottom to top are for chromatography at 1, 0 M NaCl (—); 2, 0.05 M NaCl (—); 3, 0.2 M NaCl (- - -); 4. 0.4 M NaCl (—); and 5. 1.0 M NaCl (•••); all five buffers contained 0.02 M sodium phosphate and 0.02% sodium azide, pH 7.6.

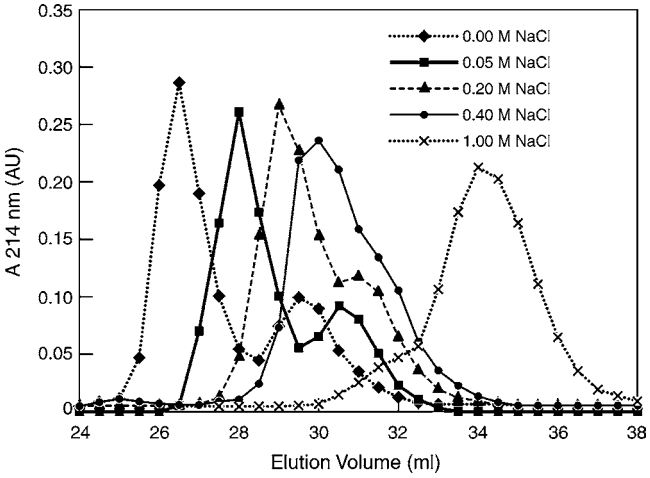

Figure 5. Composite of Superose 12 column chromatograms of purified red kidney bean α-amylase inhibitor (RKB αAI) in several ionic strength buffers. The buffers contained 0.2 M sodium acetate buffer, pH 5.2, 0.02% NaN_3, plus various concentrations of NaCl (as noted) (see **Table 2**). Glass column size: 1 × 30 cm.

mental parameters were identical for the two Superose 12 chromatographies. The chromatograms in the two buffers showed similar patterns with two exceptions. First, the inhibitor activity peak (marked with x−x) was larger in sodium acetate/acetic acid buffer (**Figure 3B**) than in sodium phosphate buffer (**Figure 3A**). This was due to some inactive protein overlap in **3B**. Second, all the peaks, except the active peak of RKB αAI (at 28.0 mL in **Figure 3A** and 31.5 mL in **Figure 3B**), were eluted at the same elution volume in sodium phosphate and sodium acetate buffers. These results indicate that the elution time and yield of RKB αAI activity were influenced by nature of buffer and pH used. Most likely, this is due to some noncovalent interaction of RKB αAI with the matrix of the column.

Ionic Strength Effect in Determining Molecular Weight on Superose 12 Column. The effect of changes of ionic strength of the buffer on purified RKB αAI and five standard MW proteins (cytochrome *c*, Kunitz soybean trypsin inhibitor, chicken egg white ovalbumin, bovine serum albumin, and human transferrin with respect to time of elution (V_n/V_o) from the same Superose 12 column were investigated. The five buffers contained 0.02 M sodium phosphate, 0.02% sodium azide, and 0.00, 0.05, 0.20, 0.40, or 1.00 M NaCl, pH 7.6. These results are summarized in **Table 2** and **Figure 4**. If there were no effect of ionic strength on the results, all five points for each protein would lie on the same descending line for V_n/V_o versus log molecular weight (*1*). However, the V_n/V_o values for all five proteins at 0.0 and 0.2 M NaCl increased with increasing NaCl concentration (**Table 2**). One might conclude that the standard curve at 0.4 and 1.0 M NaCl, with V_n/V_o being nearly identical, would give the correct molecular weight for other proteins. Unfortunately, this was not the case as shown for RKB αAI (**Table 2**) and by the data in **Figures 4** and **5**. The calculated values for RKB αAI molecular weight are shown in **Table 2**. The V_n/V_o numbers for the standard proteins increased when the NaCl concentration of the buffer increased.

The V_n/V_o (molecular weight indicator) for RKB αAI (MW of 49.0 kDa) also depended on the NaCl concentration of the buffer. The higher the NaCl concentration in the buffer, the higher the volume of buffer required to elute RKB αAI (**Figure 5**). The plateauing of V_n/V_o at a NaCl concentration of 0.2−0.4 M observed for the first four standard proteins (**Table 2**) did not occur for RKB αAI (**Figure 4**). At 0 M NaCl, the apparent MW was 14.0 kDa; at 0.05 M the apparent MW was 30.7 kDa; then the apparent MW decreased at 0.2 M (22.9 kDa), 0.4 M (16.3 kDa) and 1 M (<14 kDa) NaCl (**Table 2**). The standard curves used were at the same ionic conditions as those used for the RKB αAI. On the basis of these data, two effects appear important for determining observed molecular weights of proteins on Superose 12 columns. At 0 and 0.05 M NaCl, there appears to be an ionic interaction of RKB αAI with the Superose 12 matrix. Even at 0.05 M NaCl, the observed molecular weight was too low (30.7 versus 49.0 kDa expected). Above 0.05 M NaCl, the RKB αAI appeared to interact hydrophobically with the Superose 12 matrix as a result of increasing NaCl concentration ("salting out"). Note also that at 0−0.4 M NaCl, there is evidence of a second smaller component in RKB αAI (**Figure 5**), which was not present from Sephadex G-100 column chromatography (**Figure 1**) and was not observed by gel electrophoresis. We have no explanation for this, as the RKB αAI was pure by other criteria.

Therefore, we conclude from these results that the correct molecular weights of RKB αAI, WKB αAI, horse heart cytochrome *c*, Kunitz soybean trypsin inhibitor, ovalbumin, bovine seum albumin, and human transferrin cannot be obtained by Superose 12 chromatography. The molecular weight of WKB αAI determined by Sephadex G-100 chromatography (*1*) and by the Hedrick and Smith method (*9*) compared well with the molecular weight determined by other methods (see **Table 3**).

When Are Correct Molecular Weights of Mature Proteins Known? The simple answer is when the same molecular weight is obtained by two or more methods based on different principles. As shown in **Table 3**, all six methods gave similar but not exactly identical molecular weight for the WKB αAI (*13*). The best method for molecular weight determination is the LATOFMS. The cDNA sequence of amino acids gives a precise molecular weight for the nascent molecule, but post-translational modification of the protein can change the molecular weight. MALLS gives very good results for large molecular weight proteins, but not <50.0 kDa proteins (**Table 3**).

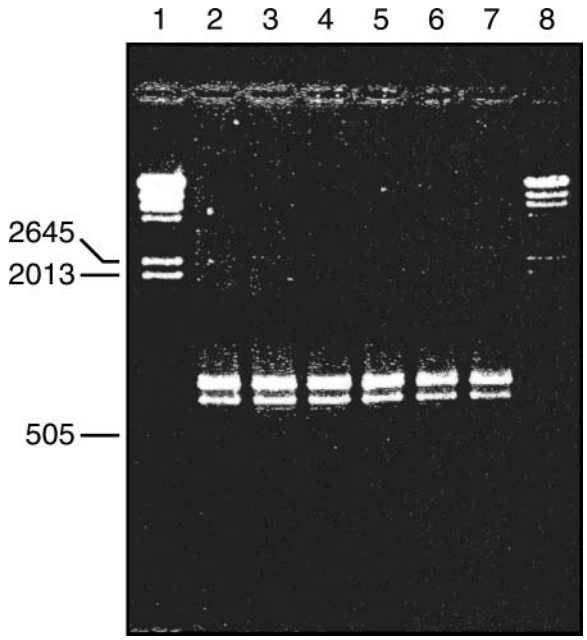

Figure 6. PCR analysis of genomic DNA purified from various *Phaseolus* genotypes: lane 1, nucleotide MW markers; lane 2, white kidney bean (WKB cv. WKB); lane 3, WKB cv. 858; lane 4, red kidney bean (RKB), Food Coop.; lane 5, RKB (Safeway); lane 6, RKB cv. RKB; lane 7, black bean (BB); lane 8, nucleotide MW markers. Ethidium bromide-stained 2.0% agarose gel shows PCR amplification products generated using primer pairs that were designed on the basis of the conserved terminal 5′ and 3′ coding regions of phytohemagglutinins (PHA-E, PHA-L), αAI, and arcelin I. DNA molecular weight markers are indicated (in base pairs) on the left (from ref *13*). The bottom bands are the genes of the six α-amylase inhibitors (αAIs) listed. The bands immediately above the αAIs are the genes for the lectins. The numbers on the left side are for nucleotide molecular weight markers.

The gene molecular weight is about the same for white kidney bean cv. 858 and red kidney bean α-amylase inhibitors, along with four other α-amylase inhibitors from *Phaseolus vulgaris* beans (**Figure 6**). The amino acid sequence of the inhibitor RKB αAI has not been determined. The genes for the following α-amylase inhibitors have been sequenced: white kidney bean, αAI-4; black bean, αAI-5; and greensleeves, αAI-1. Each mature protein has 223 amino acid residues (*13*). WKB αAI-4 and BB αAI-5 differ in sequence by change in 12 amino acid residues, while GS αAI-1 and BB αAI-5 differ in sequence by change in 7 amino acid residues.

Further complexity is found when proteins have subunits and one or more units of carbohydrate, lipid, metal ions, cofactors, etc. Also, there are more than 135 ways by which proteins collectively can be posttranslationally modified; a single protein generally will not use more than four to six of these ways.

The following example using WKB αAI is chosen to illustrate the complexity of biological synthesis and the difficulty of experimentally understanding what nature has made (*13*). In the case of WKB αAI, a 30.7 kDa pre-pro-polypeptide is synthesized as specified by the cDNA code (the gene). The N-terminal pre-segment of the polypeptide (21 amino acid sequence; 2.37 kDa) is removed by a specific protease to give the pro-polypeptide (MW 28.4 kDa). An internal peptide bond of the pro-polypeptide is hydrolyzed by a second specific protease to give an α-peptide (MW 11.4 kDa) and a β-peptide (MW 16.6 kDa). Two α-peptides and two β-peptides associate to form a tetrameric protein, which is then glycosylated (12.2% carbohydrate covalently attached). The active mature WKB αAI has a molecular weight of 56.7 Da. The molecule is an $\alpha_2\beta_2$ protein (*13*). The small α subunit contains 25.6% carbohydrate (18 glucose equivalents), while the large β subunit contains 0.94% carbohydrate (0.97 glucose equivalents). The percentage of carbohydrate varies somewhat based on the maturity of the bean and perhaps other factors. The total weight of the molecule is 56.714 kDa, as determined by laser-assisted time-of-flight mass spectrometry (*13*). It is now very easy to determine the molecular weight of a sequence of amino acid residues encoded by a gene, but often it is more difficult to determine the true molecular weight and architecture of a mature protein following posttranslational modification.

NOTE ADDED AFTER ASAP

An error in the abstract in the original posting of July 16, 2004, has been corrected July 21, 2004.

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