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Determination of protein concentration for protein-protein conjugates using ultraviolet absorption

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Abstract

The present study reports a method to determine the total protein concentration or concentration of a protein of interest in a protein-protein conjugate using ultraviolet absorption, after determining the molar ratio of proteins in the conjugates, from which an extinction coefficient can be calculated. A Microsoft Excel solver-based template using amino acid analysis data was developed for determining the molar ratio. The percent mass of each protein in the conjugate is calculated from the amino acid composition data using the least squares method in the Microsoft Excel solver function, and the percent mass is converted to molar portion of each protein using corresponding molecular weight. A molar ratio is obtained by dividing the molar portion of protein 1 by the molar portion of protein 2. A weighted extinction coefficient is calculated using the molar ratio, and the total protein concentration is determined using ultraviolet absorption at 280 nm. The accuracy of the method was verified using mixtures of known proteins. The present study provides a rapid, simple and accurate method for determining protein concentration in protein-protein conjugates.

Keywords

Concentration of protein-protein conjugate; molar ratio; Microsoft Excel solver; Amino acid analysis; least square analysis

1. Introduction

Conjugating a poorly immunogenenic compound to a highly immunogenic carrier protein to increase immunogenicity is a common practice in biological science field and has broad applications. Our previous studies demonstrated that significantly higher antibody titers could be achieved when Pfs25 or Pfs28, leading malaria transmission-blocking vaccine candidate antigens, were conjugated to the outer-membrane protein complex (OMPC) of *Neisseria meningitidis* serogroup B (Wu et al., 2006) or a recombinant nontoxic

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Pseudomonas aeruginosa ExoProtein A (rEPA) (Qian et al., 2007 and 2009). An accurate assessment of the concentration of a conjugated protein is essential to downstream investigations. The most critical step in the determination is to assess the molar ratio of protein-protein conjugates, which can be used to calculate the extinction coefficient of the conjugate. A number of methods have been developed to estimate the molar ratio of protein-protein conjugates, including radioactively labeled protein (Green et al., 1982), sodium dodecyl sulphate (SDS) electrophoresis (Jones et al., 1989), spectrophotometric method (Jones et al., 1989; Sashidhar et al., 1994), matrix-assisted laser desorption/ionization time of light (MALDI-TOF) mass spectrometry (Pakarinen et al., 2002), and capillary electrophoresis (Safi et al., 2007). However, the ratios determined by these methods were rough estimates and may not suitable for the accurate measurement of the protein concentration of a conjugate.

It appears that amino acid analysis is the most accurate method for determining the molar ratio of protein in protein-protein conjugates. In 1989, Antoni and Presentini reported a DOS- and least-squares-based method for the determination of molar ratios of two different proteins in conjugates using the results of amino acid analysis. Shuler and co-workers presented a comprehensive Microsoft Excel- and least-squares-based method to determine the ratios of small peptides to keyhole limpet hemocyanin (KLH) using amino acid analysis (Shuler et al., 1992). As technology rapidly evolves, the program written in BASIC language for the VAX 750 computer described in Antoni and Presentini's paper is no longer suitable for today's applications; and the method developed by Shuler depends on the method used to calculate protein composition from amino acid analysis data, which requires extensive verification.

In this communication, we present a simple and accurate method by which the molar ratio of protein-protein conjugates can be determined by a Microsoft Excel solver-based template using amino acid analysis data. The total protein concentration in the conjugate and the concentration of the individual protein components can be accessed using calculated extinction coefficients (Pace et al., 1995). The accuracy of this method was verified by calculating the molar ratios in known mixtures of proteins. This method should have general applications where the protein concentration of protein-protein conjugates must be estimated.

2. Material and Method

2.1 Antigen and carrier proteins

The recombinant *Pichia pastoris* expressed Pvs25 (MacDonald and Narum, unpublished), Pfs28 (MacDonald and Narum, unpublished), and AMA1-FVO (Kenedy et al 2002) proteins, as well as the *Escherichia coli* expressed ExoProtein A (rEPA) (Qian et al 2007) protein were manufactured with methods developed at the Laboratory of Malaria Immunology and Vaccinology (LMIV), National Institute of Allergy and Infectious Diseases, National Institutes of Health, the protein concentrations were determined by ultraviolet absorption at 280 nm. BSA was purchased from Thermo Fisher Scientific.

2.2 Protein mixture preparation

The known molar ratio of protein mixture were prepared according Table 1. The Mass % of protein 1 and Protein 2, the experimentally prepared molar ratio of protein 1/protein 2 were also summerzed in Table 1.

2.3 Amino Acid Analysis

The amino acid composition was determined by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT). The samples were hydrolyzed in vacuo for 16 hours at 115°C in 100 μl of 6N HCl/0.2% phenol (with 1 nmole norvaline/100 μl as an internal standard) to digest the protein into free amino acids. After hydrolysis, the HCl was dried off in a vacuum-centrifuge and the resulting amino acids dissolved in 100 μl of 0.02N HCl (with 2 nmole taurine/100 μl as a second internal standard). Amino acid analysis was carried out on a Hitachi L-8900 PH Amino Acid Analyzer which used an ion-exchange column with pH and temperature gradients to separate the amino acids and post-column derivitization with ninhydrin for detection at 570 nm and 440 nm. EZChrome Elite (for Hitachi) software was used to operate the analyzer and collect and analyze the data.

2.4 Selection of amino acids used in the calculation

Fourteen amino acids were used in our calculation. During HCl hydrolysis, asparagine is converted to aspartic acid and glutamine to glutamic acid; therefore, these amino acids were reported as aggregate Asx and Glx values. Cysteine, tryptophan, threonine and serine recoveries are typically low, methionine may be partially oxidized, and proline quantitation is often inaccurate due to interference from cysteine, so those amino acids were excluded from the calculation.

2.5 Calculation of the molar ratio of protein mixture

Table 2 is a Microsoft Excel template for molar ratio calculation. The percent composition of each amino acid in each protein (or conjugate) was obtained by dividing the experimental nmol of the amino acid of protein 1 (X1), protein 2 (X2), or conjugate (Y) with total nmol (Σ) of X1, X2, or Y to produce normalized X1 (protein 1), X2 (protein 2) and Y (conjugate), respectively. Y represents the experimentally determined percent composition of an amino acid in a conjugate. The theoretical percent composition (′Υi) of an amino acid was calculated by formula: $'\Upsilon i = (X1_i \times Z1) + (X2_i \times (1-Z1))$, where i represents an amino acid and Z1 represents the percent mass of protein 1 which is produced when $\Sigma\,(\mathbf{Y}-'\Upsilon)^2$ is the smallest as determined by the Microsoft solver (see Appendix). Subsequently, the percent mass of protein 1 (Z1) is converted to molar portion in the conjugate by dividing the percent mass by its molecular weight. The molecular weight can be either the intact or modified molecular weight, i.e. molecular weight calculated by amino acid residues used. The molar portion of protein 2 was calculated by dividing the percent mass (100% − percent mass of protein 1) with its molecular weight (intact or modified molecular weight). The molar ratio of protein 1 to protein 2 was obtained by dividing molar portion of protein 1 with molar portion of protein 2. This template can be easily used for determining molar ratios of proteins in a conjugate.

2.6 Calculation of the Extinction Coefficient of a conjugate

After determining the molar ratio of a conjugate, the molar absorption coefficient at 280 nm for the protein conjugate was calculated by the equation described by Pace et al. (1995), i.e. ε280 (M^{-1} cm^{-1}) = (no. of Trp) × (5,500) + (no. of Tyr) × (1,490) + (no. of paired Cys) × (125). The weighted extinction coefficient (ml mg^{-1}) was calculated by dividing ε280 with the molecular weight of the protein conjugate.

The weighted extinction coefficient of conjugate was also calculated by the extinction coefficient of each protein obtained from ExPASY. The weighted extinction coefficient of conjugate = [(extinction coefficient of protein antigen × milligram of protein antigen) + extinction coefficient of carrier protein] ÷ (1 + milligram of protein antigen).

3. Results

3.1 Analysis of protein mixture with known ratio

The nominal molar ratios (the known molar ratios of the protein mixtures) and the calculated molar ratios by the method developed in the present study are summarized in table 3. The average percent accuracy was 94.55 ± 3.36 % (intact molecular weight) or 96.39 ± 1.70 % (modified molecular weight) when calculated with the present method, demonstrating that the method developed in the present study is able to accurately determine the molar ratio of protein conjugate.

3.2 Calculation of Extinction Coefficient of conjugate

Extinction coefficient of a protein sequence is readily available from ExPASY site. The weighted extinction coefficient of a conjugate can be calculated using the extinction coefficients of the two components after their ratio is determined. It can also be calculated directly using Pace's equation once the amino acid composition of the conjugate is known. The weighted extinction coefficients of 8 conjugates were calculated using these two methods. As anticipated, results were almost identical with the average variation ± standard deviation of 0.065 % ± 0.070 % (data not shown). These tiny variations could come from the roundup of decimal of the extinction coefficient of each monomer protein and suggest that direct usage of Pace's equation is a better approach to calculate the extinction coefficient of the protein conjugate.

3.3 Calculation of concentration of protein interested

The concentration of total protein in conjugate can be obtained by dividing the ultraviolet absorption reading at 280 nm (UV_{280}) with the extinction coefficient or directly from amino acid analysis with the adjustment of missing residues.

The concentration of protein of interested can be calculated by the following equation:

$$\text{Concentration of protein of interested} = \{(\text{molar ratio} \times \text{molecular weight of protein interested}) \div [(\text{molar ratio} \times \text{molecular weight of protein interested}) + \text{molecular weight of carrier protein})]\} \times \text{concentration of total protein}.$$

4. Discussion

Although the previously reported method is able to calculate the peptide to protein molar ratio in a conjugate, the accuracy of the calculation depends on the method used to calculate protein composition from amino acid analysis data. The theoretical amino acid composition of the peptide was used for the calculation in the original paper (Shuler et al., 1992). However, the results were not satisfactory when theoretical amino acid composition of proteins were used to calculate the known ratio of protein mixtures in our study. The accuracy was improved when experimentally determined amino acid compositions were used. However, there are several ways to calculate the experimental amino acid composition and they require extensive verification. The present study used the raw data in nmole of amino acid, directly obtained from amino acid analysis, eliminating the error that could occur during converting nmol to residue number, as done in the previously reported method. We also found that the Microsoft Excel Solver, a what-if analysis tool for optimization, was very powerful and accurate in finding an optimal % mass of protein 1. In addition, the template for the ratio calculation (Table 2) calculates the ratios in a concise and reproducible manner.

Post-translational modifications are common in proteins. During amino acid analysis, most of the post-translational modifications are removed from the amino acids by hydrolysis and therefore, are not quantitated by the analysis. For the modifications may not be removed (or be partially removed) by hydrolysis, a more accurate result can be obtained if the theoretical amino acid sequence or composition is known along with a molecular weight that includes all the modifications. In fact, except for glycosylation, many post-translational modifications only account for a small percentage of the total protein molecular mass and thus, do not have significant effect on amino acid analysis data.

Fourteen amino acids were used in our calculation. However, an amino acid is eliminated from the calculation if it underwent a chemical modification or used as a stabilizer in the solution. In many cases, a protein conjugate is composed of a mixture of conjugates with a wide range of protein to protein ratios. The method described in the present study is capable of determining the average ratio of the protein conjugate. Additional purification steps may be required if a more accurate ratio of certain conjugate product needs to be determined.

Amino acid analysis can be time consuming and expensive. A quick and easy way to estimate the molar ratio of protein in a conjugate can be achieved using a spectrophotometry. According to the Beer-Lambert law, $A_{280nm} = €IC$; where € is the molar absorption coefficient (M^{-1} cm^{-1}), I is the path length (cm), and C is the protein concentration (M). The molar absorption coefficient $€ = A_{280nm}/IC = A_{280nm}/C$ when I is 1cm. In routine practice, researchers may choose to use weighted extinction coefficient. i.e. absorbances (E) for 0.1% solutions (=1 g/l), $E = A_{280nm}/C$ (protein concentration in mg/mL) when a 1cm thickness cuvette is used. The protein concentration (C) of the conjugate can be determined by a number of methods and therefore, E can be experimentally determined. If the composition of each monomer protein is known, the weighted extinction coefficient at different protein to protein ratios can be obtained from ExPASY or directly from Pace's equation and a standard curve of weighted extinction coefficient for the conjugate vs. the molar ratio can be generated. The molar ratio of protein 1 to protein 2 can thus be calculated from the equation of the standard curve. As expected, the accuracy of this estimation is lower than that determined by the method developed in the present study.

In summary, a rapid, simple and accurate method was developed for determination of the molar ratio of proteins in protein-protein conjugates using amino acid analysis data. This allows for the calculation of protein concentration using calculated extinction coefficients. This method should have general applications to determining protein concentration in any peptide-peptide, peptide-protein and protein-protein conjugate.

*This worksheet is designed to analyze as many residues as possible, the user may choose residues to use and/or delete the residues that may result in low accuracy.

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6. Appendix

Install solver tool to Microsoft Excel as instructed by manufacturer.

Specific parameters to run the solver

Set Target Cell: target cell to calculate the sum of $(\mathbf{Y}-\hat{\Upsilon})^2$, as in Table 2, it is I13

Equal to: check “min” option

By Changing Cells: change cell as % mass of protein 1, as in Table 2, it is D16

Subject to Constraints: leave blank

Select a solving method: GRG Nonlinear

Run the solver

Once configured, click the Solve button in the Date tab to run the solver on the Excel spreadsheet and a message “Solver found a solution. All constraints and optimality condition are satisfied” appears. Click on “ok” button, the molar ratio of protein 1 to protein 2 appears automatically.

Table 1

Preparation of the known molar ratio of protein mixture

Protein 1	Protein	Conc. of protein 1 (mg/mL)	Conc. of protein 2 (mg/mL)	Volume of protein 1 in mixture (μL)	Volume of protein 2 in mixture (μL)	% Mass of Protein 1[a]	% Mass of Protein 2[b]	Molar ratio of protein 1/protein 2[c]
Pfs25	BSA	0.770	0.752	37.5	12.5	75%	25%	1.111
				25	25	51%	49%	3.332
				12.5	37.5	25%	75%	9.995
AMA1-FVO	EPA	0.437	0.841	5	15	15%	85%	0.187
				10	10	34%	66%	0.562
				15	5	61%	39%	1.685
Pvs 25	EPA	0.690	1.265	15	10	45%	55%	2.650
Pvs28	EPA	1.308	1.225	10	10	52%	48%	3.396

[a] % Mass % of protein 1=[(volume of protein 1 in the mixture × concentration of protein 1)÷(volume of protein 1 in the mixture × concentration of protein 1 + volume of protein 2 in the mixture × concentration of protein 2)] × 100

[b] % Mass of protein 2=[(volume of protein 2 in the mixture × concentration of protein 2)÷(volume of protein 1 in the mixture × concentration of protein 1 + volume of protein 2 in the mixture × concentration of protein 2)] × 100

[c] Molar ratio of protein 1/protein 2=(% Mass of protein 1 ÷ molecular weight of protein 1)÷(% Mass of protein 2 ÷ molecular weight of protein 2)

Table 2

Template in Microsoft Excel format for molar ratio calculation*

	A	B	C	D	E	F	G	H	I
		Experimental data (nmole)			Normalized data (%)			ϒ̂	(Y−ϒ̂)²
		X1	X2	Y	X1	X2	Y		
1	Asx	2.5886	1.5470	2.2239	0.1999	0.1188	0.1582	0.1579	7.2703×10^{-8}
2	Glx	2.0869	2.4461	2.4603	0.1612	0.1879	0.1750	0.1750	3.5468×10^{-9}
3	Gly	1.2052	0.5589	0.9185	0.0931	0.0429	0.0653	0.0671	3.1088×10^{-6}
4	Ala	0.3728	1.2923	0.9363	0.0288	0.0993	0.0666	0.0653	1.5886×10^{-6}
5	Val	1.6232	1.0057	1.4244	0.1254	0.0772	0.1013	0.1004	7.6615×10^{-7}
6	Ileu	0.8871	0.3855	0.6789	0.0685	0.0296	0.0483	0.0483	4.3462×10^{-9}
7	Leu	0.8676	1.7725	1.4488	0.0670	0.1361	0.1030	0.1029	3.2973×10^{-8}
8	Tyr	0.3534	0.5299	0.4975	0.0273	0.0407	0.0354	0.0342	1.2879×10^{-6}
9	Phe	0.2359	0.7842	0.5361	0.0182	0.0602	0.0381	0.0400	3.5186×10^{-6}
10	His	0.5207	0.3794	0.4873	0.0402	0.0291	0.0347	0.0345	3.2240×10^{-8}
11	Lys	2.0096	1.6924	2.0124	0.1552	0.1300	0.1431	0.1421	9.5056×10^{-7}
12	Arg	0.1957	0.6254	0.4369	0.0151	0.0480	0.0311	0.0322	1.2362×10^{-6}
13	Σ	12.9467	13.0193	14.0613	1.00	1.00	1.00	1.00	1.2603×10^{-5}
14	Modified molecular weight of protein 1[b]			15551.20	Molar portion of protein 1[c]				3.0967×10^{-5}
15	Modified molecular weight of protein 2[b]			53361.28	Molar portion of protein 2[d]				9.7156×10^{-6}
16	% mass of protein 1[a]			0.4816	Molar ratio of Protein 1/Protein 2[e]				3.1873

X1, X2 and Y columns represent the experimentally determined amino acid composition of protein 1, protein2 and conjugate in nmol, respectively; **X**1, **X**2 and **Y** columns represent the normalized amino acid composition of protein 1, protein 2 and conjugate, respectively. Normalization was calculated using this formula (use Asx in protein 1 as example): Normalized Asx in protein 1(cell E1) = 2.5886 (cell B1) ÷ 12.9467 (cell B13) = 0.1999. ϒ̂ column represents the theoretical amino acid composition of a conjugate in % total nmol of protein and calculated as follows (use Asx as example): Asx theoretical conjugates (%) = (normalized Asx in protein 1) × (% mass of protein 1) + ((100% − (% mass of protein 1)) × (normalized Asx in protein 2) = 0.1999 (cell E1) × 0.4816 (cell D16) + (100% − 0.4816 (cell D16)) × 0.1188 (cell F1) = 0.1579 (cell H1). $(\mathbf{Y} - \hat{\Upsilon})^2$ column represents the squared difference of each amino acid between experimental and theoretical of conjugates and was calculated as follows (use Asx as example): $(\mathbf{Y} - \hat{\Upsilon})^2$ = (% of Experimentally normalized conjugate − % of theoretical conjugate)^2 = (0.1582 (cell G1) − 0.1579 (cell H1))^2 = 7.2703×10^{-8} (cell I1).

[a]% mass of protein 1 is calculated using Microsoft Excel solver.

[b] Modified molecular weight is the sum of molecular weight of amino acids residues actually used in the calculation. In this example, it is the sum of molecular weight of 14 amino acids (Asx for asparagine and aspartic acid and Glx for glutamine and glutamic acid).

[c] Molar portion of protein 1=(% mass of protein 1)÷(Modified molecular weight of protein 1)=0.4816 (cell D16)÷15551.20(cell D14)=3.0967×10^{-5} (cell I14).

[d] Molar portion of protein 2=(100%−% mass of protein 1)÷(Modified molecular weight of protein 2) =(100%−0.4816 (cell D16))÷53361.28 (cell D15)=9.7156×10^{-6} (cell I15).

[e] Molar ratio of protein1 to protein2=Molar portion of protein 1÷Molar portion of protein 2=3.0967 $\times 10^{-5}$(cell I14)÷9.7156×10^{-6} (cell I15)=3.1873 (cell I16).

Table 3

Comparison of the nominal molar ratio and the calculated molar ratio

Protein 1/Protein 2	Molar ratio of protein 1/protein 2 (nominal)[a]	Molar ratio of protein 1/protein 2 (calculated)[b]		% Accuracy[c]	
		By intact MW[d]	By modified MW[e]	By intact MW[d]	By modified MW[e]
Pfs25/BSA	1.111	1.016	1.072	91.49	96.52
	3.332	3.022	3.187	90.71	95.67
	9.995	9.731	10.267	97.36	97.27
AMA1-FVO/EPA	0.187	0.201	0.197	92.63	94.69
	0.562	0.584	0.573	95.96	97.95
	1.685	1.646	1.614	97.65	95.77
Pvs 25/EPA	2.650	2.422	2.628	91.41	99.18
Pvs28/EPA	3.396	3.370	3.195	99.24	94.07
		Average % Accuracy		94.55	96.39
		Standard deviation		3.36	1.70

[a] Known molar ratio of protein 1 to protein 2 (nominal) was obtained by preparing the known molar concentration of protein 1 and protein 2 in the same mixed sample.

[b] Molar ratio of protein 1 to protein 2 (calculated) was calculated by the method presented in Table 2 using the amino acid data.

[c] $$\%\ \text{Accuracy} = [\,100\% - |(\text{molar ratio of calculated} - \text{molar ratio of nominal})/(\text{molar ratio of nominal})|\,] \times 100\%$$.

[d] Intact molecular weight of protein 1 and protein 2 were used in the calculation.

[e] Modified molecular weight of protein 1 and protein 2, i.e. sum of molecular weight of amino acid residues actually used in the calculation were used in the calculation.