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Measurement of amino acid compositions of glycoprotein systems by gas-phase hydrolysis and reversed-phase high-performance liquid chromatography

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ABSTRACT

Interest in glycoproteins and their compositions has increased in recent years. Work described in this report illustrates the use of an amino acid analysis protocol involving gas-phase hydrolysis and reversed-phase high-performance liquid chromatography of glycoprotein systems at microgram levels. In other amino acid analysis protocols the problem of losses of amino acids of glycoproteins has been documented. These losses were due to various reactions, referred to as browning or Maillard reactions, which yielded a residue from which amino acids were not recoverable.

In our work, three glycoprotein systems are examined: ovalbumin, sICAM-1, and bovine serum albumin —which is naturally unglycosylated, but is spiked with about 30% saccharides. In all three cases, the compositional agreement between the molar ratio of amino acids determined empirically and that predicted is greater than 90%. Thus it is shown that the adverse effects of Maillard-type reactions are avoided, and the presence of carbohydrates causes negligible interferences with amino acid analysis performed under the conditions described herein.

INTRODUCTION

Amino acid analysis is an extremely valuable technique for characterization and quantitation of polypeptides [1–5]. The technique consists of three basic steps frequently performed in the following order: hydrolysis of peptide bonds to liberate amino acids, derivatization of the liberated amino acids, and separation of the derivatized amino acids. The three steps are carried out with numerous chemistries including hydrolysis by hydrochloric acid or methanesulfonic acid in the gas or liquid phase; derivatization by ninhydrin, *o*-phthalaldehyde, phenylisothiocyanate, dabsyl chloride or fluorenylmethyl chloroformate; and separation by reversed-phase or ion-exchange high-performance liquid chromatography (HPLC).

Pioneered and developed within the last decade, one of the most widely used protocols of amino acid analysis begins with hydrolysis by hydrochloric acid in the gas phase, followed by derivatization by phenylisothiocyanate and then separation by reversed-phase HPLC [3,5–13]. This particular protocol is applicable to the analysis of many classes of polypeptides especially at microgram levels. However, application to the class of glycoproteins remains largely uninvestigated.

Amino acid analysis of glycoproteins demands special consideration. The potential for losses of amino acids due to browning reactions that occur during liquid-phase hydrolysis of milligrams of glycoproteins is extensively documented [1,2,14]. Briefly, browning reactions, which are also referred to as Maillard reactions, lead to brown residue called humin or melanoidins. The humin is composed of products of reactions between amino acids and saccharides, their derivatives (*e.g.*, sialic acid), or their acid-catalyzed degradation intermediates. The exact composition and molecular structure of humin remain unknown. Nonetheless it is clear that

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amino acids as well as saccharides from which humin is derived are virtually irrecoverable for most purposes, including amino acid analysis.

A complicated scheme of reactions, some in series, others in parallel, yields humin [1,2,14]. Both aldoses and ketoses can react with the amine moiety of amino acids or peptides. The nature of the reactions and products depends on moisture, temperature, and pH. For instance at a temperature greater than 100°C and pH less than 1, which occur during hydrolysis with 6 *M* hydrochloric acid, saturated and unsaturated dicarbonyl compounds as well as α-, β-unsaturated carbonyl compounds can form from the constituent saccharides of the glycoproteins and react with amino acids. At a higher pH, such as pH greater than 4, which occurs during the removal of the hydrochloric acid used for hydrolysis, intact reducing sugars or their degradation products can react with amino acids. In either acidic condition, humin can be produced, rendering amino acids (and saccharides, for that matter) essentially irretrievable for consequent analysis.

In view of the potentially adverse browning reactions, the research reported herein addresses the integrity of amino acid analysis that incorporates hydrolysis of glycoprotein by hydrochloric acid in the gas phase, derivatization by phenylisothiocyanate, and separation by reversed-phase HPLC. Three glycoprotein systems at the microgram level are examined. Two are naturally glycosylated proteins, ovalbumin and sICAM-1 (soluble intercellular adhesion molecule 1), the latter being a potent and specific *in vitro* inhibitor of rhinovirus infection [15–17]. The third glycoprotein system is naturally unglycosylated bovine serum albumin which is spiked with saccharides roughly simulating the carbohydrate content of sICAM-1. Compositional agreement, a mathematical parameter that quantifies the correspondence of the amino acid molar ratio determined empirically to that predicted, is used to demonstrate that the presence of saccharides causes negligible interference with the accuracy of amino acid analysis performed under the conditions described.

EXPERIMENTAL

Apparatus

Hydrolysis of proteins and derivatization of amino acids were performed using the Waters Picotag workstation module (Waters/Millipore, Milford, MA, USA). The liquid chromatographic system consisted of a Waters 680 automated gradient controller module, Waters 590 pump, Waters 510 pump, Waters WISP automatic sampling device, Dupont Instruments column compartment (DuPont, Wilmington, DE, USA) and a Kratos spectroflow 757 UV–VIS absorbance detector (Kratos, Ramsey, NJ, USA). Chromatographic data were analyzed by a Hewlett-Packard HP 1000-A600 processor laboratory automation system (Hewlett-Packard, Palo Alto, CA, USA) with HP 3357 software and by a Vectra personal computer (Hewlett-Packard) with custom-designed software for the measurement of amino acids. A Waters Picotag column, C_{18}, 15 × 0.39 cm, was used for HPLC. Pyrex 9820 (50 × 6 mm) culture tubes were from Corning (Corning Glass, Corning, NY, USA) and reaction vials from Waters.

Chromatographic solvents

Picotag eluent A and Picotag eluent B [13] were used for mobile phase solvents. Picotag diluent was used for solubilizing derivatized amino acid samples prior to HPLC injection.

Amino acid analysis solvents

Phenol was ACS-reagent grade (Aldrich, Milwaukee, WI, USA). Water was HPLC grade (Milli Q and Milli-RO, Millipore, Milford, MA, USA). Triethylamine, hydrochloric acid (6 *M*, constant boiling) and phenylisothiocynate were all purchased from Pierce (Rockford, IL, USA). Methanol was HPLC grade high purity (Burdick & Jackson, Musk egon, MI, USA). Ethanol was Absolute-200 proof (AAPer Alcohol & Chemical Co., Shelbyville, KY, USA).

Proteins, saccharides and amino acids

Amino acid standard H was purchased from Pierce. D(+)-Galactosamine (cell culture reagent grade), D(+)-mannose, and N-acetyl-D-glucosamine were all purchased from Sigma (St. Louis, MO, USA). Albumin from bovine serum, and ovalbumin from chicken egg-grade V, were both purchased from Sigma. sICAM-1 was produced recombinantly in Chinese hamster ovary cells and was purified by tangential flow ultrafiltration and

affinity liquid chromatography [18]. All glycoprotein systems were dissolved in Dulbecco's phosphate-buffered saline.

Hydrolysis and derivatization

Hydrolysis and derivatization were performed largely according to Waters Picotag methodology [7,13]. Samples were pipetted into 50 × 6 mm tubes previously pyrolyzed at 500°C for 16 h in a muffle furnace. After the addition of sample, the tubes were placed in a reaction vial and the samples were dried. A 200-μl volume of 6 *M* HCl containing 1% (v/v) liquefied phenol was added to the bottom of the reaction vial. The vial was purged with nitrogen, sealed, and placed in the oven compartment of the workstation for hydrolysis, which was carried out for 0.5 to 2.0 h at 150°C. One reaction vial was used per each duration of hydrolysis. After the appropriate time of hydrolysis, the reaction vial was removed and the tubes were taken out and transferred to a clean reaction vial. The hydrolyzed samples were dried, neutralized by delivering to each tube 20 μl of a solution of methanol–water–triethylamine (2:2:1), and vortexed. The neutralized samples were dried, derivatized by adding to each tube 10 μl of a solution of methanol–water–triethylamine–phenylisothiocyanate (7:1:1:1), and vortexed. The samples were allowed to stand for 20 min at room temperature. The derivatized samples were dried, and unless otherwise noted, each was reconstituted with 200 μl of Picotag diluent and 4 μl were injected onto the HPLC column.

Chromatography

Reversed-phase HPLC of the derivatized amino acids was performed using a Waters Picotag column and Picotag eluents A and B. Gradients were taken from ref. 13. Absorbance was at 254 nm and 0.05 a.u.f.s. Injection volume was 4 μl. The amino acids were identified by retention times and quantified by areas, as compared to those of standards. Customized software, prepared by the Analytical Sciences and Management Information Systems Departments of Boehringer Ingelheim Pharmaceuticals, was used for these identifications and quantifications. In addition, the software calculated an experimental molar ratio of amino acids. This calculation was relative to the predicted or known number of moles of a selected amino acid present in one mole of test polypeptide.

Data collected from the detector was transferred to an analog-to-digital (A/D) converter (118652A, Hewlett-Packard), then to the 1000-A600 processor, and finally to a Vax computer (Digital Equipment Corporation, Maynard, MA, USA) over a LAN ethernet. The customized software ran off of the Vectra personal computer and was written in Turbo Pascal (Borland International, Scotts Valley, CA, USA). The Vectra computer was connected to the VAX computer also over a LAN ethernet and used the VAX computer as a virtual disc drive.

Calculations

Compositional agreement (*CA*), a mathematical parameter defined as follows, was calculated to quantify an overall accuracy of each amino acid analysis.

$$CA = 100\% \times [N_p - (\sum|n_e - n_p|)]/N_p$$

where: N_p = the predicted (known) total number of moles of all amino acids in one mole of polypeptide; n_e = the empirically determined number of moles of a specific amino acid in one mole of polypeptide; n_p = the predicted (known) number of moles of a specific amino acid in one mole of polypeptide.

Compositional agreement as given above is apparently equivalent to per cent correct composition [19] and inversely related to per cent error [5,12] reported previously. These indexes all serve with comparable utility the same substantive purpose of providing a simple and quantitative parameter to assess an overall accuracy of an amino acid analysis.

Semantically, however, we prefer the nomenclature compositional agreement, because it has greater general applicability. Both per cent correct composition and per cent error are nomenclature that imply that the true amino acid content of a sample is definitively known. Frequently there are samples (*e.g.*, synthetic peptides, recombinant fusion proteins) for which the amino acid content is intended but not definitively known. Even for samples of pure standards of polypeptides, it could be argued that the true content of amino acids is not definitely known because of the ubiquitous nature and often unknown magnitude of background amino acid contaminants [5,10,12,13,19]. Thus because it implies less presupposition about the true content of amino acids in a sample, the term compositional agreement is used throughout this report.

RESULTS AND DISCUSSION

Bovine serum albumin as a control

Bovine serum albumin was selected as a control because of its lack of glycosylation. Hence the possibility of interference of carbohydrates with amino acid analysis was precluded. Over the course of several weeks amino acid analyses were performed on a total of 14 samples of bovine serum albumin. Highly reproducible chromatograms similar to that presented in Fig. 1A were obtained.

Measurements of amino acid compositions were also reproducible and generally within 15% agreed with predicted compositions, as illustrated by a typical example given in Table I. Such agreement, representative of the other 13 analyses, was reasonable for a single analysis and was consistent with results reported earlier for commercially available unglycosylated proteins of high purity [5,12,19,20]. For each of the 14 analyses of bovine serum albumin, compositional agreement was also calculated. Values ranged from 88 to 95% and averaged 91%, which again was comparable to results reported previously [5,12,19,20]. Thus, values of approximately 90% for compositional agreement typified amino acid analyses for which there was no possibility of problems attributable to the presence of saccharides.

Glycoprotein systems

The three glycoprotein systems that were analyzed and compared to the control were chicken egg ovalbumin, sICAM-1 and bovine serum albumin to which saccharides were spiked. As ovalbumin and sICAM-1 were naturally glycosylated, no saccharides were added. Ovalbumin was composed of approximately 4% (w/w) saccharides [21,22], while sICAM-1 was composed of approximately 30% saccharides [15]. sICAM-1 was produced recombinantly in Chinese hamster ovary cells, which secreted the sICAM-1 in a soluble form [18]. This form simulated the five extracellular domains of ICAM-1, a naturally occurring, membrane-bound glycoprotein which was identified as a human cellular receptor for the subgroup of rhinoviruses known as major groups [16]. sICAM-1 was found to be a potent and specific *in vitro* inhibitor of rhinovirus infection [16].

The glycoprotein system containing bovine serum albumin included 30% (w/w) saccharides to simulate roughly the carbohydrate content of sICAM-1 [15,17,23]. Mannose, N-acetylglucosamine and galactosamine, in equal amounts (*i.e.*, 10% each) were the added saccharides, because they were common to various types of O-linked and N-linked oligosaccharides [24], and the sequence of amino acids of sICAM-1 exhibited eight potential sites (Asn-X-Ser or-Thr) of N-linked glycosylation [15].

A total of five samples of ovalbumin in three amounts (50, 100 and 185 μg) were hydrolyzed and analyzed (Fig. 1B) for amino acid composition. Compositional agreement ranged from 87 to 89% and averaged 88% (Table II). Also at three levels (35, 70 and 125 μg), a total of eight samples of bovine serum albumin spiked with saccharides were hydrolyzed and analyzed (Fig. 1C, Table II). Compositional agreement ranged from 88 to 95% and averaged 92%. Hydrolysis and analysis of sICAM-1 at 42 μg and 184 μg gave an average compositional agreement of 92% (Fig. 1D, Table II). For each of the three glycoprotein systems and for each of the seven levels spanning 35–185 μg, compositional agreement was equivalent to that obtained with the control of bovine serum albumin that was not spiked with saccharides (*cf.* above and Table I). Therefore the potentially adverse effects of browning reactions were negligible.

Variation of hydrolysis time

The study of the effects of browning reactions on amino acid analysis of sICAM-1 was extended to encompass various durations of hydrolysis, which have been recommended [1,2,19,25,26] in order to assess as accurately as possible amino acid composition. Rates and extents of hydrolytic cleavage of peptide bonds have depended on the specific amino acids participating in the bonds [1,2,25,26]. Also, the stabilities of amino acids liberated from peptide bonds have depended on the specific amino acid. Thus for sICAM-1 hydrolysis durations of 0.5, 0.75, 1.0, 1.5 and 2.0 h were employed, with recoveries given in Table III for each amino acid.

As expected, levels of Tyr, Ser and Thr (Table III) decreased 15–40% with increased time of hydrolysis, ostensibly because of a greater susceptibility of these three amino acids to progressive hydrolytic destruction [1,2,25,26]. Levels of Glx and Asx decreased 30–50% as well with increased duration of

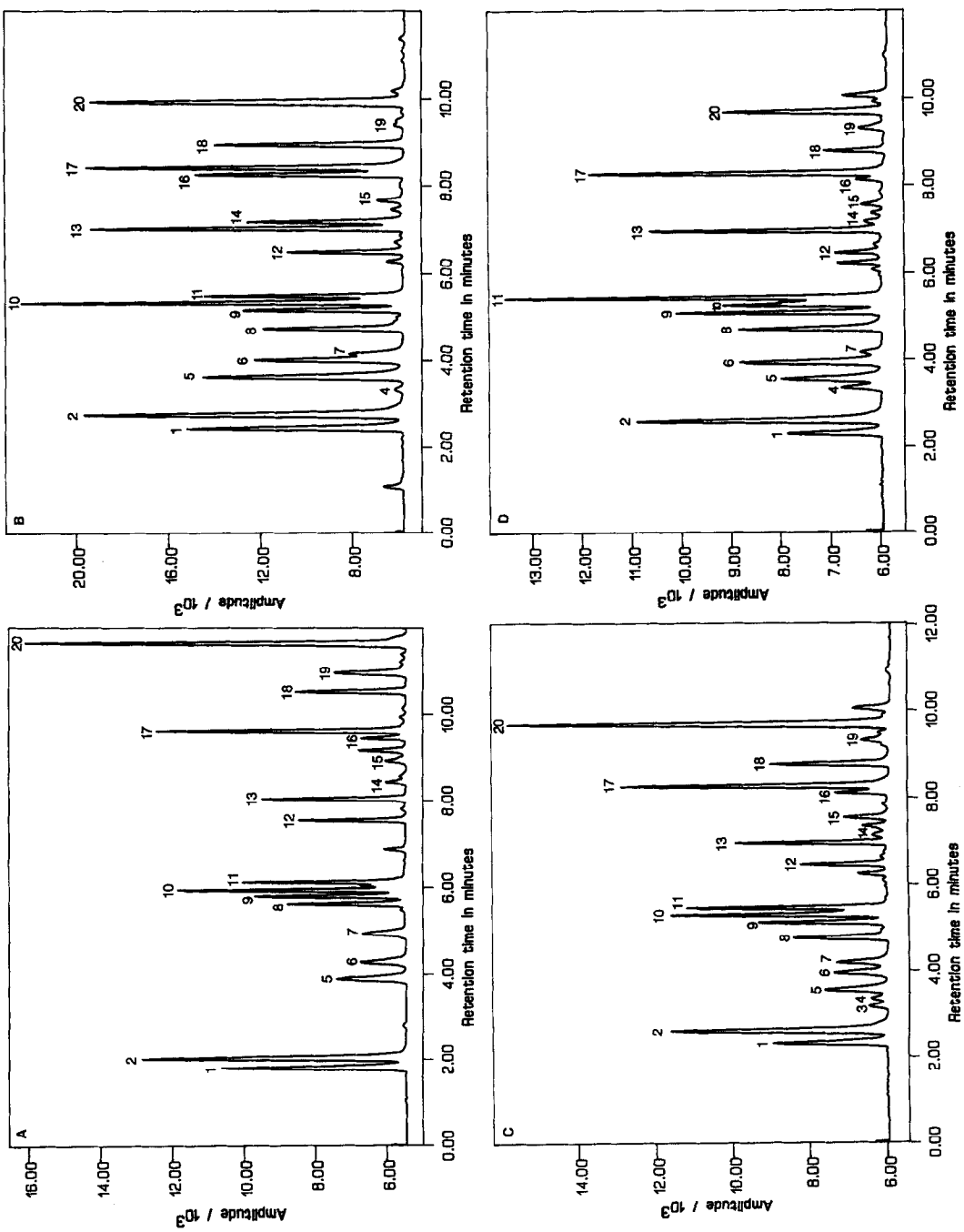

Fig. 1. Typical amino acid analysis chromatograms of (A) bovine serum albumin, (B) ovalbumin, (C) bovine serum albumin, spiked with saccharides, and (D) sICAM-1. All were hydrolyzed for 1 h at 150°C. A was generated using conventional Picotag methodology which includes a column oven temperature of 38°C [13]. B, C and D were generated using the gradient, the addition of extra triethylamine to eluent A, and the column oven temperature (42°C) recommended in advanced Picotag methodology for amino sugars [13]. Peaks: 1 = aspartic acid (Asp); 2 = glutamic acid (Glu); 3 = galactosamine (GalN); 4 = glucosamine (GlcN); 5 = serine (Ser); 6 = glycine (Gly); 7 = histidine (His); 8 = arginine (Arg); 9 = threonine (Thr); 10 = alanine (Ala); 11 = proline (Pro); 12 = tyrosine (Tyr); 13 = valine (Val); 14 = methionine (Met); 15 = cysteine (Cys); 16 = isoleucine (Ile); 17 = leucine (Leu); 18 = phenylalanine (Phe); 19 = impurity; 20 = lysine (Lys).

TABLE I

TYPICAL AMINO ACID ANALYSIS OF BOVINE SERUM ALBUMIN (WITHOUT SACCHARIDES)

Amino acid[a]	Measured (pmol)[b]	n_e[c]	n_p	$\lvert n_e - n_p \rvert$
Asx	924	59	48	11
Glx	1280	81	75	6
Ser	404	26	28	2
Gly	271	17	15	2
His	267	17	17	0
Arg	372	24	23	1
Thr	487	31	34	3
Ala	722	46	46	0
Pro	484	31	28	3
Tyr	314	20	19	1
Val	484	31	36	5
Met	71	5	4	1
Cys	—[d]	—[d]	35[e]	—[d]
Ile	182	12	14	2
Leu	912	58	61	3
Phe	409	26	26	0
Lys	966	62	59	3
Trp	—[d]	—[d]	2[e]	—[d]
Total $CA = 92\%$	—	—	$N_p = 533$	$\sum \lvert n_e - n_p \rvert = 43$

[a] Definitions of abbreviations are given in the key in the legend of Fig. 1. In addition Asx is the sum of asparagine and aspartic acid, and Glx is the sum of glutamine and glutamic acid.

[b] 10 μg of bovine serum albumin in 10 μl of water were hydrolyzed at 150°C for 1 h, derivatized, reconstituted in 100 μl, and 10 μl were injected.

[c] Calculated relative to the predicted number of residues of phenylalanine (26).

[d] Not determined.

[e] Disregarded in the calculation of N_p and compositional agreement.

hydrolysis, possibly because of longer exposure to metal ions [8,19,20,27]. Indeed in our experiments no precautions (*e.g.*, addition of EDTA) were taken to offset losses of Asp and Glu due to interactions with ionic calcium, magnesium, sodium, and potassium, which were present in the sample matrix after sICAM-1 was purified prior to amino acid analysis [18,23].

Interestingly levels of Leu, Ile and Val also decreased 30–40% with increased duration of hydrolysis (Table III). This was surprising. The hydrophobic side chains of Leu, Ile and Val were reported to shield proximal peptide bonds from hydrolytic attack by hydronium ions [2]. Longer times of hydrolysis were therefore suggested [1,2,25,26] in order to achieve greater cleavage of peptide bonds relatively resistant to hydrolysis and, consequently, to liberate more completely Leu, Ile, Val and adjacent amino acids sharing these bonds. Thus, that recoveries of Leu, Ile and Val of sICAM-1 decreased with increased hydrolysis time was not anticopated. It was possible that Leu, Ile and Val preferentially reacted with saccharides in a time-dependent fashion and that longer hydrolysis times resulted in greater losses. However inspection of the sequences of amino acids in the vicinities of the eight potential glycosylation sites of sICAM-1 revealed no particular preponderance of Leu, Ile or Val [15].

From the data presented in Table III, an optimized determination of amino acid composition of sICAM-1 was made. A molar ratio of amino acids was calculated by taking for each amino acid the largest amount measured as a function of hydrolysis time. This approach exploited the highest recovery achieved for every residue [2]. The molar ratio of amino acids determined empirically in sICAM-1 (Table IV) closely approximated that predicted. For 13 of 16 amino acids the agreement between experimental and predicted was within 15%. Indeed compositional agreement was 92%. Thus the potentially adverse effects of browning reactions were not evident.

From the data presented in Table III, compositional agreements were calculated for each of the individual durations of hydrolysis. For the longer hydrolyses (*e.g.*, 1.5 and 2.0 h), the compositional agreements were poor, *i.e.*, less thn 80%, evidently due to losses of Tyr, Ser, Thr, Glx, Asx, Leu, Ile and Val, as mentioned above. However, the compositional agreements achieved for the shorter hydrolysis times were greater than 90%, which was equivalent to that obtained in the optimized determination of molar ratio depicted in Table IV. Thus an accuracy of measurement as good as that obtained in the extensive hydrolysis study was attainable with single short hydrolyses, although this was not predictable, making it prudent to conduct the hydrolysis time study [25,26].

The study of the effects of browning reactions on amino acid analysis was extended further to include various durations of hydrolysis of bovine serum albumin spiked with saccharides. Trends similar to those illustrated in Table III were obtained for

TABLE II

COMPOSITIONAL AGREEMENT OF GLYCOPROTEIN SYSTEMS

	Spiked (μg)			Saccharide content (%)	Compositional agreement[a]
	Mannose	N-Acetylglucosamine	Galactosamine		
Ovalbumin (185 μg)[b]	0	0	0	4	87, 87 ($n=2$)
Ovalbumin (101 μg)	0	0	0	4	89 ($n=1$)
Ovalbumin (50 μg)	0	0	0	4	88, 89 ($n=2$)
Bovine serum albumin (125 μg)	20	20	20	32	93, 91, 91 ($n=3$)
Bovine serum albumin (70 μg)	10	10	10	30	95, 95 ($n=2$)
Bovine serum albumin (35 μg)	5	5	5	30	88, 89, 92 ($n=3$)
sICAM (184 μg)	0	0	0	*ca.* 30	93 ($n=1$)
sICAM (42 μg)	0	0	0	*ca.* 30	91, 91 ($n=2$)

[a] Cysteine and tryptophan were disregarded.
[b] Amount hydrolyzed at 150°C for 1 h and derivatized.

TABLE III

AMINO ACID ANALYSIS OF sICAM-1 BY VARIOUS TIMES OF HYDROLYSIS

Amino acid[b]	Measured (pmol)[a]				
	Hydrolysis time (h)				
	0.5	0.75	1.0	1.5	2.0
Asx	440	385	252	266	218
Glx	727	700	610	548	483
Ser	370	337	296	282	251
Gly	403	381	356	339	335
His	61	60	55	57	49
Arg	302	302	295	292	270
Thr	406	428	392	403	368
Ala	335	333	354	346	335
Pro	505	504	527	531	521
Tyr	98	87	79	89	58
Val	494	464	394	374	334
Met	46[c]	46	43	45	41
Ile	59	59	48	51	47
Leu	608	593	500	478	429
Phe	128	120	108	114	104
Lys	220	203	185	188	162

[a] Given values are averages of duplicate amino acid analyses, results of which differed for each entry no more than 10%. For each analysis 33 μg of sICAM-1 in 10 μl of phosphate buffered saline were hydrolyzed at 150°C and derivatized.
[b] Definitions of abbreviations are given in the footnotes of Table I and in the key in the legend of Fig. 1.
[c] Single determination rather than an average.

nearly all amino acids, particularly Tyr, Ser, Thr, Asx, Glx, and even Leu, Ile and Val (data not shown). A molar ratio of amino acids was calculated by taking for each amino acid the maximum amount determined as a function of hydrolysis time. The molar ratio determined empirically was in good agreement with that predicted (Table IV), as the compositional agreement was 90%, and for 12 of 16 amino acids experimental values were within 15% of predicted values.

All of the studies described above served to demonstrate that no significantly deleterious effects of browning reactions on overall amino acid analysis of glycoprotein systems as performed were encountered. Possibly insufficient carbohydrate reactant was present, although both sICAM-1 and bovine serum albumin spiked with saccharides contained substantial fractions (30%) of carbohydrate. Alternatively hydrolysis in the gas phase instead of the liquid phase may have effectively immobilized the potential reactants (*i.e.*, amino acids and saccharides and their derivatives) to the wall of the hydrolysis tube such that it became impossible for them to diffuse. Therefore, they could not contact one another to react.

This hypothesis was consistent with several earlier reports. For instance in order to minimize the loss of amino acids during liquid-phase hydrolysis of amino acid analysis, a dilute solution of glycoprotein was

TABLE IV

OPTIMIZED DETERMINATION OF MOLAR RATIOS OF AMINO ACIDS

Amino acid[a]	sICAM-1		Bovine serum albumin spiked with saccharides	
	Experimental[b]	Predicted	Experimental[b]	Predicted
Asx	35.4	34	43.9	48
Glx	57.8	61	68.6	75
Ser	29.4	30	25.1	28
Gly	31.8	30	16.8	15
His	4.8	5	16.0	17
Arg	24.1	26	22.5	23
Thr	32.7	43	28.0	34
Ala	26.5	25	45.9	46
Pro	40.6	40	38.5	28
Tyr	7.7	8	15.8	19
Val	36.9	43	31.0	36
Met	3.7	2	4.4	4
Cys	—[c]	14[d]	—[c]	35[d]
Ile	4.7	7	10.9	14
Leu	48.2	53	54.8	61
Phe	10.0	10	26.0	26
Lys	17.3	17	56.6	59
Trp	—[c]	5[d]	—[c]	2[d]

[a] Definitions of abbreviations are given in the footnotes of Table I and in the key in the legend of Fig. 1.
[b] Calculated relative to the predicted number of residues of phenylalanine.
[c] Not determined.
[d] Disregarded in the calculation of N_p and compositional agreement.

recommended [2]. Presumably, a low concentration of glycoprotein makes it less likely that potential reactants, such as amino acids and saccharides or their derivatives, will collide in solution; without collision, there can be no reaction [2].

More recent reports also implicated the importance of concentration and collision [28,29]. In both of these papers excellent compositional agreements were obtained for glycoproteins, even though the amino acid analysis employed liquid-phase hydrolysis. Precolumn derivatization and reversed-phase HPLC were used. Indeed in one of the reports, phenylisothiocyanate was the derivatizing agent [28]. In the other paper, the derivatizing agent was 4-dimethylaminoazobenzene-4′-sulfonyl chloride [29]. Significantly, common to both papers was the microgram levels of glycoprotein hydrolyzed. Moreover, in the latter paper [29], recoveries of amino acids were decreased only when concentration of glucose was increased ten-fold. These findings induce intriquing questions pertaining to mechanisms of browning reactions, which further work should help to elucidate.

CONCLUSIONS

Amino acid analysis that employs the widely used protocol of hydrolysis by hydrochloric acid in the gas phase, followed by derivatization with phenylisothiocyanate and then separation by reversed-phase HPLC, is applied successfully to glycoprotein systems at the microgram level. Three glycoprotein systems are examined: ovalbumin which is comprised naturally of about 4% carbohydrate, sICAM-1 which is comprised naturally of about 30% carbohydrate, and bovine serum albumin which is naturally unglycosylated but is spiked with about 30% saccharides to simulate roughly the carbohydrate content of sICAM-1. In all three cases the compositional agreement between the molar ratio of amino acids determined empirically and that predicted is typically greater than 90%. Such excellent compositional agreement is comparable to that obtained in the amino acid analysis of proteins lacking carbohydrate. Thus it is concluded that, in amino acid analysis as performed, the adverse effects

of Maillard-type reactions are avoided and the presence of carbohydrates causes no serious problems with the determinations of molar ratios of amino acids in glycoprotein systems.

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