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Improved binary high performance liquid chromatography for amino acid analysis of collagens

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Abstract

Resolution of complex amino acid mixtures obtained through hydrolysis of collagen (Type I) was achieved using pre column derivatisation and an octadecyl HPLC column. The use of a simple binary gradient in contrast to the usual complex gradient systems requiring precise control over mobile phase compositions ensured reproducible results. The selectivity offered by the stationary phase for the amino acids under the conditions used in this study makes the procedure less stringent for obtaining good resolution between amino acids differing slightly in their hydrophobicity.

Keywords: Amino Acid analysis, HPLC, Collagen

1 Introduction

Collagen is the most abundant protein in animal tissues and exists in various forms from skin and bone to basement membrane of capillaries. Collagens belong to a family of fibrous proteins, which provide the major biomechanical scaffold for anchorage of macromolecules and cell attachment. The distinctive nature of collagen poses a challenge for characterizing this protein using amino acid analysis. Collagen is characterized by a unique triple helical structure comprising of three polypeptide chains (alpha chains). In order for these chains to be wound around one another to form a super helix, the smallest amino acid, glycine (Gly) (having only a hydrogen atom as a side chain) must be regularly spaced at every third residue along each chain resulting in the repeating amino acid sequence, Gly-Xaa-Yaa. Xaa and the Yaa can be any amino acid, although they are often the imino acids proline (Pro), which is present in higher than normal concentrations in collagen, and hydroxyproline (Hyp). Another modified amino acid, hydroxylysine (Hyl), is also present in collagen, where it functions to stabilize side-by-side packing of collagen molecules through covalent aldol cross-links [1,2]. Collagen differs from other fibrous proteins (elastin, keratin and reticulin) by its high content of Hyp, Pro and Hyl, by its low tyrosine (Tyr) and sulphur content and by the absence of tryptophan (Trp). It also has a significantly higher content of polar amino acids than other fibrous proteins [1]. The amino acid composition of collagen varies between species, which is manifested in differences in the shrinkage temperature (the temperature at which sudden shrinkage in collagen fibrils occur as it is gradually heated in an aqueous medium) of collagen between species. This is primarily due to the number of Hyp residues present which in turn determines the number of water mediated hydrogen bridges formed, influencing the stability of collagen molecules. Because Gly is present in every third position in the amino acid sequence, it gives an enormous response during amino acid analysis. Added to the unusual distribution of a number of amino acids present in collagen, such as Pro, Hyp and Hyl, a careful selection of both stationary and mobile phases is required to provide a good selectivity and to prevent overlapping of closely eluting peaks.

Separation and quantitation of acid hydrolysates of proteins and peptides is a common technique used by biochemists. Various methods are in use for quantitation of amino acids. Post column derivatisation of free amino acids with ninhydrin, after separation by ion-exchange columns was introduced by Moore and Stein nearly 50 years ago [3]. Since then, improvements in technology have resulted in reduced analysis times, increase in sensitivity of analysis and the ability to analyze limited sample amounts concomitant with increased use of reversed-phase chromatography and pre column derivatisation. Various pre-column derivatising agents such as ortho-phthalaldehyde (OPA) [4], phenylisothiocyanate (PITC)[5], 9-fluorenylmethyl chloroformate (FMOC-Cl) [6], dansyl [7] and 4-dimethylazobenzenesulfonylchloride (Dabsyl-Cl)[8], have their own disadvantages. OPA fails to react with secondary amino acids and the derivatives formed are unstable. Multiple derivatives are formed with FMOC-Cl and Dabsyl-Cl along with significant interference from the reagent itself. More recently a simple derivatisation procedure with highly sensitive and stable 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) has made this analysis a more robust technique. Sensitive fluorescence detection of highly stable urea adducts formed from the reaction of amino acids with AQC and good derivatising efficiency in presence of common buffer salts and detergents, provides an advantage over other derivatising agents [9].

In this paper we have reported an improvement over a previous method used for the AQC derivatised amino acid analysis of collagen by using a more convenient binary gradient and a Phenomenex Gemini C18 HPLC column.

2 Materials and Methods

2.1 Chemicals

6-aminoquinoline (AMQ), Di(N-succinimydyl) carbonate (DSC), sodium acetate trihydrate, ethylenediamine tetraacetic acid (EDTA), sodium azide, boric acid, triethylamine (TEA) and acetic acid were obtained from Sigma (St. Louis, MO, USA). Amino acid standards were from Pierce (Rockford, IL, USA). Acetonitrile (MeCN) was from SDS (France).

2.2 Synthesis of AQC

AQC was synthesized according to the method described by Cohen *et.al.* [9]. Briefly, 12 mM DSC was dissolved in dry acetonitrile and heated to reflux. 10 mM AMQ (in dry acetonitrile), was then slowly added to the refluxing solution over 30 mins. The solution was refluxed for another 30 mins after which it was hot filtered and concentrated to half its volume using a rotary evaporator (Buchi, Rotavapor-R). The solution was kept at 4 ºC for 24 hrs. The crystals obtained were washed twice with cold acetonitrile, then recrystallized from acetonitrile to obtain off-white crystals with a melting point between 210-215 ºC.

2.3 Preparation of standards and samples

2.5 mM stock solutions of Hyp and Hyl were prepared in water. 40µl each of Pierce H amino acid standard, Hyp and Hyl stock solutions were mixed with 880µl of water to give a final concentration of 0.1 mM per amino acid. 10µl of the standard solution was taken and derivatised.

Collagen (Type I) samples (120 mg) were placed in clean, acid washed and pyrolyzed 6 X 50 mm test tubes and dried under vacuum. 200µl of 6 N HCl was then added to the samples along with a crystal of phenol. The tubes were sealed under vacuum and hydrolysed at 110 ºC for 24 hrs. After hydrolysis, the tubes were dried in a vacuum desiccator and the samples dissolved in 100µl of borate buffer.

2.4 Derivatisation of amino acids

10µl of the amino acid standards and 2µl of collagen samples were were each made to pH 8.8 with borate buffer to a total volume of 80µl. Derivatives of the amino acids were formed by the addition of 20µl of 10 mM AQC (3 mg/ml in acetonitrile).

2.5 Separation of AQC derivatised amino acids

Amino acid analysis was performed using a Dionex HPLC system equipped with a P580 pump and ASI-100 auto-sample injector. 10µl of samples were injected on to a Phenomenex 150 x 4.6mm Gemini C18, 5µ HPLC column protected with a guard column. The analysis was performed at 34 ºC using a tailored gradient run. Mobile phase A contained sodium acetate (140 mM), sodium azide (7.5 mM), disodium EDTA (0.26 mM) and Tri-ethyl amine (20mM), in water, and was titrated to pH 5.05 with phosphoric acid. Mobile phase B contained 60% MeCN in water (v/v).

The eluting peaks were monitored using a fluorescence detector (Dionex RF 2000) with excitation wavelength set at 245 nm and emission at 395 nm. At the end of each run the column was washed with 100%B for 10 mins followed by re-equilibration with 1%B for 20 mins.

3 Results and Discussion

Hydrolysis of collagens generates complex amino acid mixtures. The presence of a very high proportion of Gly and unique amino acids like Hyp and Hyl make resolution of these amino acids a challenging task. Cohen *et.al* have reported that the gradient slope used in a binary elution for separation of collagen amino acid standard mixture is critical, as a steep slope results in poor resolution of Hyp and the reagent hydrolysis product, [9].

The presence of diastereomers of Hyl, eluting in a crowded portion of the chromatogram close to valine (Val), does not leave much room to vary the gradient composition of the mobile phase, thus compromising baseline resolution of these amino acids [10]. Due to the enormous response of Gly in collagen hydrolysates, a good resolution between Gly and His is also required since histidine (His) elutes close to Gly and has a much lesser response.

Previous studies carried out by Cohen et.al. have also demonstrated that the separation between AMQ and Hyp is highly pH dependent; the resolution of each being compromised when mobile phase pH is increased. However, a low pH affects the resolution of Gly and His peaks, making them elute closer to each other [10]. A shallow gradient addressed most of these problems and the critical baseline resolution between AMQ and Hyp and between Gly and His was achieved. Also acidic amino acids such as aspartic acid (Asp) and glutamic acid (Glu) showed good resolution from other polar amino acids under acidic conditions. Use of low pH mobile phase buffer, however, posed another problem; Hyl eluted in a region of the chromatogram with non-polar amino acids and was poorly resolved. This poor resolution of Hyl could not be improved with the type of reversed phase column being used. Cohen *et.al.* used

a complex quaternary gradient system to solve these problems: the initial low pH of the eluent was rapidly changed to a higher pH in the middle of the run to increase the selectivity of the non-polar amino acids [10].

In this study, collagen (Type I) was analyzed as it is the most abundant collagen compared to other types. The analytical procedure has been simplified by avoiding a quaternary gradient system and by taking advantage of the unique selectivity of the Phenomenex Gemini HPLC column. C18 columns were chosen over C8 or C4 column because of their greater selectivity which resulted in overall better separation of individual amino acids (results not shown). Separations of amino acids were performed with Zorbax Eclipse XDB-C18 and Gemini C18 columns. The critical separation of His from Gly was easily achieved with Gemini C18, under the run conditions used, mainly because of the higher surface area (375 m^2/g) and higher carbon loading (14%) compared to the Zorbax column, which had a surface area (180 m^2/g) and a carbon load (10%). The higher surface area and carbon loading of the Gemini column resulted in greater selectivity of the amino acids. The pH of the mobile phase A, was maintained at 5.05 through out the run. Instead the proportion of the acetonitrile in the buffer was varied. The gradient profile used in this modified method is shown in Table 1.

The selectivity offered by the Phenomenex Gemini column is ideal for this particular separation of amino acids, and by using this simplified gradient profile, very good resolution of the AMQ peak and that of Hyp was obtained. The diastereomers of Hyl also were well resolved from adjoining amino acid peaks. Baseline resolution of nearly all the amino acids could be reproducibly obtained. During the analysis, however, it was observed that in order to obtain a good resolution between the hydrophilic amino acids especially, Asp and Ser, the column had to be initially conditioned with the mobile phase A for about an hour, failing which the baseline resolution of these amino acids could not be achieved (Fig. 2). Re-equilibration in mobile phase A, in subsequent runs could however, be dropped to 10 mins without adversely affecting the resolution of Asp and Ser. The optimized separation of collagen amino acids is shown in Fig. 1.

With fluorescence detection, 300 pmol of total amino acid concentration could be analyzed without compromising on the resolution of Gly and His. Also, because of very high content of Gly in collagen, more than 300 pmol produced a response for the Gly peak beyond the limit of the detector. Lower amounts, however, made quantitation of the Hyl peaks difficult.

Table 1: Gradient profile used for collagen analysis.

Time (min)	Flow (ml/min)	%B	Curve
Initial	1.00	0	*
0.5	1.00	1	Linear
15	1.00	4	Linear
23	1.00	7	Linear
28	1.00	8	Linear
46	1.00	11	Linear
52	1.00	21	Linear
71	1.00	31	Linear
76	1.00	35	Linear
79	1.00	40	Linear
80	1.00	100	Linear

Due to high selectivity of the amino acids obtained using the Gemini column the steepness of the gradient could be increased from 46 mins onwards, to elute the hydrophobic amino acids in a shorter elution time and the critical resolution between Hyl1, Tyr and Hyl2 was easily achieved. Table 2 lists the amino acid composition of collagen isolated from sheepskin. The results are in good agreement with previously published data.

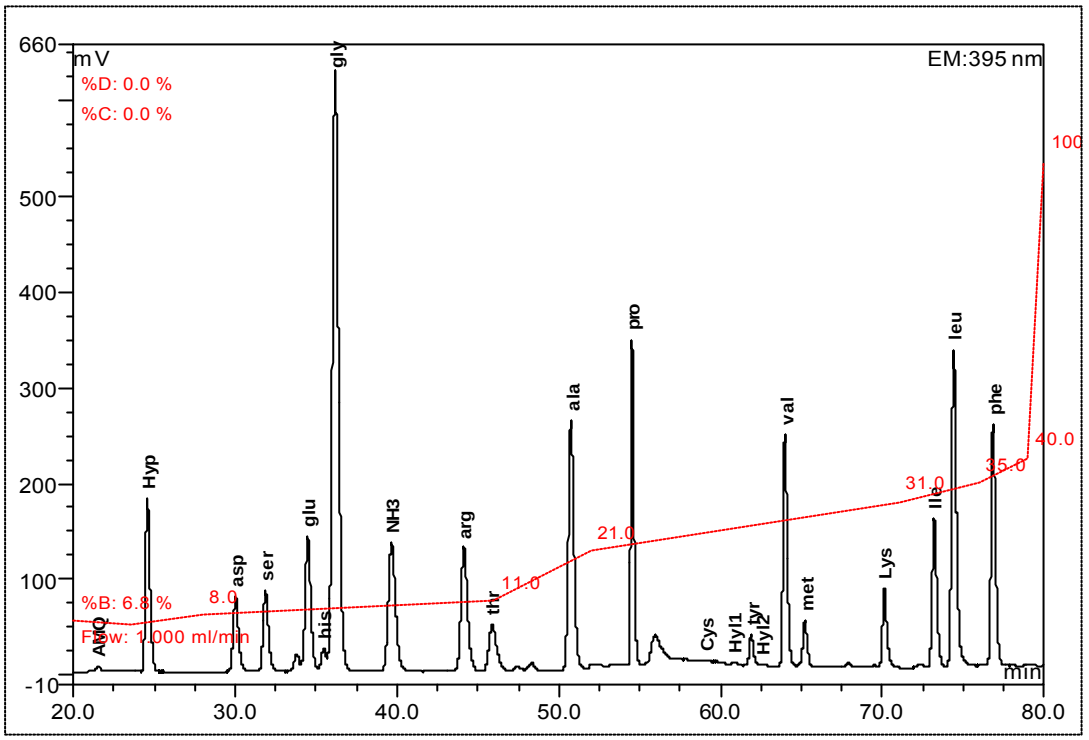

Fig. 1: Separation of collagen hydrolyzate using a gradient elution.

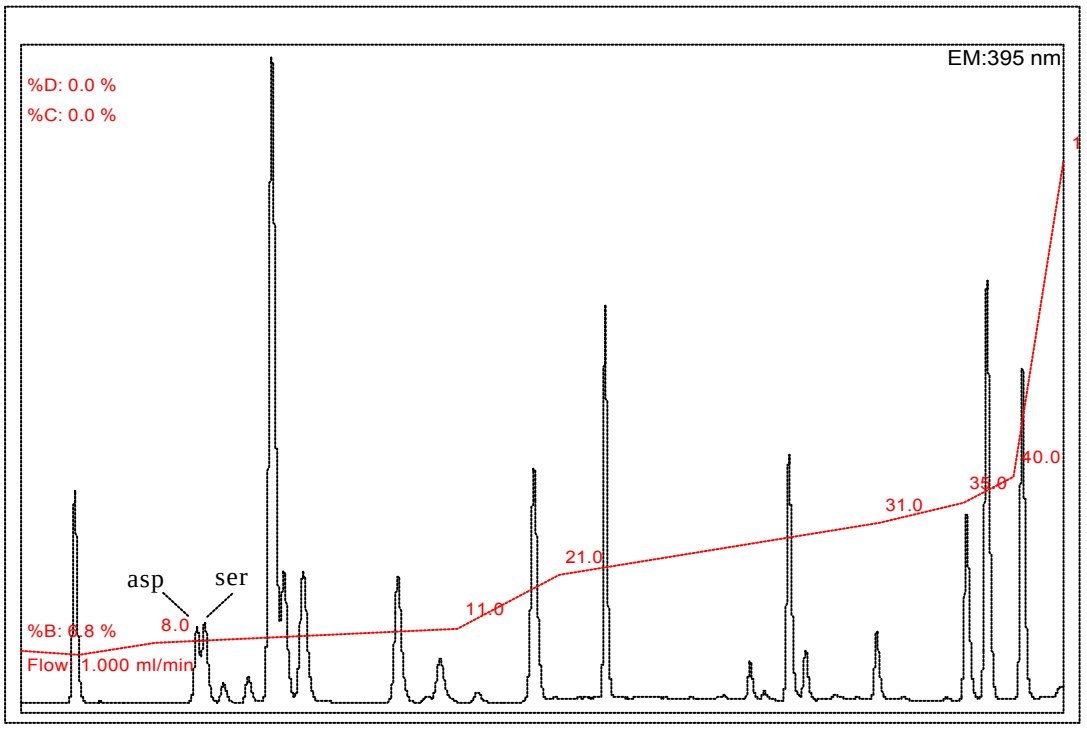

Fig. 2: Poor resolution of Asp and Ser due to insufficient equilibration with mobile phase A.

Table 2: Collagen amino acid composition

Amino acid	Experimental data[1]	Amount Reproducibility (%RSD; *n=3*)	Retention time Reproducibility (%RSD; *n=3*)	Data reported by Wandelen et.al. Ref [10]	Data reported by Hubbard et.al. Ref [11]
Hyp	173	1.30	0.285	107	97
Asp	36	1.99	0.121	44	43
Ser	29	1.71	0.049	34	25
Glu	61	2.05	0.064	85	89
His	4	1.55	0.055	3	2
Gly	333	2.33	0.076	316	333
Arg	47	1.87	0.058	54	50
Thr	20	2.45	0.039	26	23
Ala	79	1.73	0.045	99	103
Pro	91	0.62	0.012	113	120
Cys	ND[2]	ND[2]	ND[2]	2	ND[2]
Hyl	16[3]	9.02	Hyl1 0.021 Hyl2 0.027	24	20
Tyr	6	0.92	0.022	3	2
Val	20	0.83	0.034	19	18
Met	6	1.95	0.043	6	10
Lys	24	1.13	0.058	16	15
Ile	14	1.04	0.005	9	9
Leu	28	1.97	0.006	26	26
Phe	17	2.35	0.005	13	13

[1] Amino acid expressed per 1000 residues, [2] Not determined, [3] Value given as a sum of two Hyl isomers

Differences in some of the amino acids in sheep collagen from those reported from other sources are due to differences in the source of collagen. As expected, Gly was the most abundant amino acid. Hyp, Pro, Ala and Glu were also present in high proportions.

Pre-column derivatisation of amino acids with AQC not only simplified the derivatisation procedure but also produced reproducible results (Table 2). The use of very sensitive fluorescence detection of the derivatives provided subpicomole sensitivity of detection without any interference from the derivatising agent.

Formation of Hyp, a post-translational modification of collagen chains, is far more extensive in collagen than in other proteins. Quantification of derivatised imino acids such as Hyp is used extensively to determine the tissue collagen content and collagen bisosynthesis [12, 13, 14]. Since these studies are only concerned with the resolution of Hyp and or Pro from the derivative amino acid mixture, isocratic elution is often used to quantitate Hyp. The method described in this study can also be adapted for analysis of tissue Hyp levels, in other words collagen synthesis, using a much shorter run time of about 28 mins. Because of the large selectivity of Hyp it is not necessary to use a gradient elution to separate Hyp from the rest of the derivatised amino acids. Rather, a shorter isocratic elution of the mix would separate Hyp, allowing its quantitation.

In this study it has been clearly shown that reproducible separation of complex amino acid mixtures as those obtained through hydrolysis of collagen can be achieved using simple gradient elution.

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