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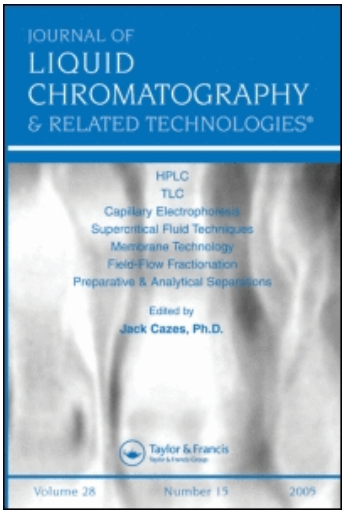

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Ion Exchange HPLC of a Marine Collagen

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Ion Exchange HPLC of a Marine Collagen

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Abstract: HPLC resolution of fibrillar collagen chains, and particularly the α_2 chain from β and γ components, has continued to provide a challenge. A single column method for the clear resolution of the denatured chains of hoki (*Macruronus novaezelandiae*) skin type I collagen is presented. The effect of changing chromatographic parameters (flow rate, loading, temperature, gradient and solvent, pH) was examined. The α_1 and α_3 chains were readily resolved under all the conditions studied. The α_2 chain was the most difficult component to resolve but also gave the largest response to changes in solvent gradient and pH.

Keywords: Collagen, Hoki, HPLC

INTRODUCTION

High performance liquid chromatography (HPLC) is a valuable tool for the study of large proteins.[1,2] However, the separation of collagen components still provides challenges, as shown by the combinations of separation modes and number of manipulations usually required to achieve resolution.[3]

Reverse phase (RP) HPLC has been explored by several groups investigating the separation of the fibrillar collagens, namely, types I, II, and III, and their cyanogen bromide (CNBr) cleavage products.[4–9]

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While separation between the individual α chains can be readily achieved, the presence of the β and γ components interferes with the complete separation of all the fractions. Neither the temperature nor the n-alkyl chain length (of the silica derivatising agent) have a significant influence on the resolution of the collagen chains under gradient elution conditions with low pH water–acetonitrile combinations.[8] Similarly, the molecular size differences between the collagen components (100,000–300,000 Da) made a relatively minor contribution to the separations.

Another published method for the separation of the fibrillar collagen components is HPLC gel permeation chromatography on a Separon Hema 10000 Glc.[10] Although a gel permeation packing was used, the authors recognise separation occurs via a mixed mode mechanism combining the hydrodynamic volume with interaction of the collagenous components and the sorbent used. Separation of α_1I and α_2I using this method is poor in the published chromatograms.[10]

Ion exchange (IE)-HPLC has also been investigated as a method for the fractionation of Type I collagen α chains and CNBr peptides.[11,12] Both groups used gel permeation in combination with IE chromatography to achieve their purification.

As part of our studies to characterise marine collagen we investigated a strong cation exchange HPLC packing (Dionex ProPac SCX-10) to separate hoki (*Macruronus novaezelandiae*) skin collagen components. In this work we report on the use of a single HPLC mode to achieve separation and how the chromatography parameters influence resolution of the hoki collagen components.

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EXPERIMENTAL

HPLC

Samples were analysed on a Dionex HPLC system with a P680 high pressure pump, a thermostated column compartment (TCC-100), and a temperature controlled ASI-100 automated sample injector. The columns used were ProPac SCX-10 analytical columns (4×250 mm) with matching guard columns.

Collagen Preparations

The two collagen preparations used during these studies were hoki skin acid soluble collagen (HS-ASC) and hoki skin collagen prepared following pepsin treatment (HS-pep).

HS-ASC

Hoki skins were scraped clean of all adhering muscle and fat, and washed in 0.05 M Tris-HCl pH 7.5 with 1 M sodium chloride (NaCl) prior to extraction in acetic acid (0.5 M). Acid soluble collagen was recovered by centrifugation and type I fibril forming collagen recovered by precipitation out of 0.5 M acetic acid with 0.83 M NaCl. The collected collagen pellet was redissolved and dialysed against 0.1 M acetic acid in preparation for freeze drying and the sample was stored at −80°C until required for HPLC analysis.

HS-pep

Hoki skins were cleaned and the acid soluble collagen was extracted as described above. Collagen was recovered from the 0.5 M acetic acid extract by precipitation using 1.4 M NaCl. The pellet was recovered by centrifugation, redissolved in acetic acid (0.5 M), and digested with pepsin (Sigma) for 8 hours at a ratio of 1:54 (enzyme to collagen), at 7.5 +/−0.5°C. Collagen was recovered by salt precipitation (1.4 M NaCl in 0.5 M acetic acid) and the pellet redissolved in 0.5 M Tris/HCl at pH 7 with 0.5 M NaCl. A further purification was carried out by reprecipitation at 3.3 M NaCl from the neutral solvent. The collected pellet was redissolved in acetic acid (0.5 M) and dialysed against acetic acid (0.1 M) before freeze drying. The sample was stored at −80°C until required for HPLC analysis.

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Sample Preparation

Samples (50 mg) were taken up in solvent A (5 mL) and put in the shaking water bath at 30°C for 15 min to solubilise and denature the protein. The samples were clarified in the bench top micro centrifuge at 13000 rpm for 10 min prior to filtration through a 0.8 μm filter. Samples were stored at 4°C between runs. The protein concentration was measured using the Biuret method[13] to confirm sample loadings.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Collected peak fractions were combined. These fractions were dialysed prior to freeze drying. Samples were taken up in a gel sample buffer and analysed on Criterion™ Precast Gels according to the manufacturer's instructions. The protein bands were visualised using Bio-Safe™

Coomassie G250 stain (Bio-Rad, New Zealand). All peaks were identified from their SDS-PAGE profiles and are labelled in the Figures.

Amino Acid Analysis

Collected peak fractions were combined, dialysed prior to freeze drying, and hydrolysed in 6 M HCl at 110°C for 24 h. After hydrolysis all amino acids were analysed using the Waters AccQTag chemistry at the Australian Proteome Analysis Facility Ltd, Macquarie University, Sydney, Australia.

RESULTS

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Collagen comprises approximately 20% of the wet weight of cleaned hoki skins. In contrast to mammalian skin sources, only a negligible amount of high molecular weight (HMW) material (>300 kD) was detectable by SDS-PAGE analysis of the acid extract of hoki skins. This is supported by the prevalence of acid soluble covalent cross links in hoki skin (unpublished data) and the similar appearance of the HS-ASC and HS-pep on SDS-PAGE gels (Figure 1). Both these collagen preparations have been analysed by IE-HPLC in this work.

The optimised HPLC separation achieved for pepsin extracted hoki skin Type I collagen is illustrated in Figure 2a. The solvent was 1 mM sodium acetate-acetic acid, pH 4.4, and the sample was eluted at 1 mL/min with a linear gradient of 0.2 to 0.52 M NaCl over 80 min at 30°C. The peaks were identified from their elution positions on SDS-PAGE (Figure 2b) and labelled according to their elution order as previously determined.[14] Peak fractions were collected and the amino acid content was determined. The grouped amino acid data shown in Table 1 support the identification of the peak fractions. The β fractions represent two α chains still associated by covalent linkages; therefore the identity of the β peaks was determined by the similarity of the amino acid profiles to those of the subunit α chains.

The effect of sample load on the separation of acid soluble hoki skin collagen was determined. Specifications supplied with the Dionex ProPac SCX-10 column indicated that depending on the specific proteins and resolution of the protein peaks, the expected column capacity would allow >100 mg of protein to be injected on this size column without loss of resolution. The results shown in Figure 3 indicate that hoki collagen has much lower loading tolerances and that approximately 0.3–0.4 mg was the optimal loading for hoki collagen samples.

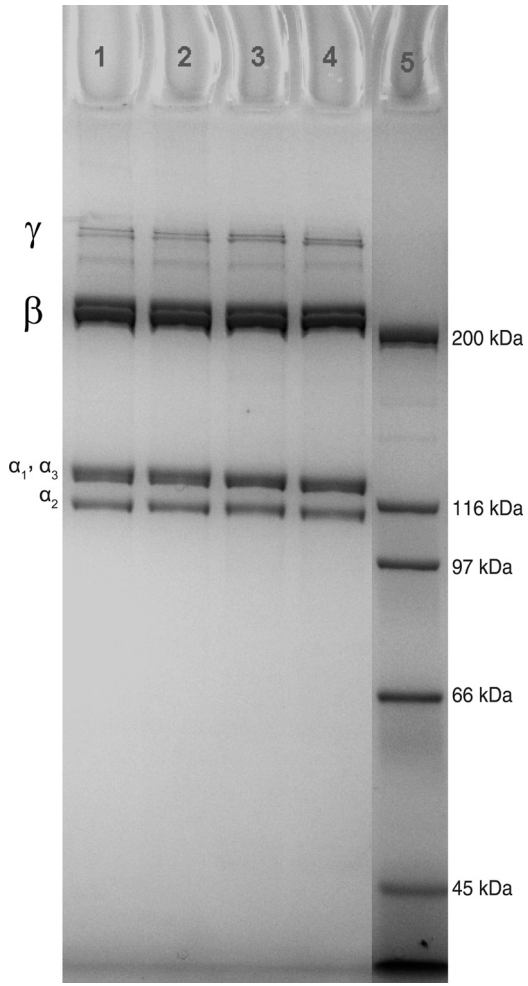

Figure 1. SDS-polyacrylamide gel electrophoresis profiles of hoki skin type I collagen extracts. Lanes 1 and 2 contain 1.5 μg of hoki skin acid soluble collagen (HS-ASC). Lanes 3 and 4 contain 1.5 μg of hoki collagen extracted after pepsin treatment (HS-pep).

The influence of temperature on the separation of the collagens was determined at 20, 25, 30, and 35°C. The column maximum is 40°C. The chromatograms for 20°C and 35°C are shown in Figure 4. The increase in retention times for the peaks is reflected in the capacity factor (k) data[15] given for all four temperatures in Table 2. It is expected that at higher temperature, more rapid mass transfer would result in enhanced chromatographic efficiencies.[1] However, we observed an increase in retention

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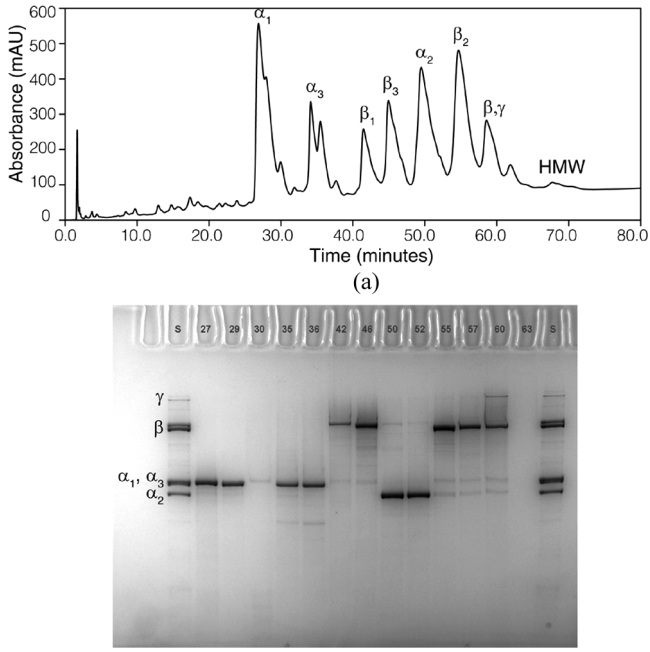

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Figure 2. Separation of denatured type I collagen chains from hoki (*Macruronus novaezelandiae*) skin by ion exchange chromatography. (a) Collagen (HS-pep) (260 μg) was chromatographed on a Dionex ProPac SCX column. The solvent was 1 mM sodium acetate-acetic acid, pH 4.4, and the sample was eluted with a linear gradient of 0.2 to 0.52 M NaCl over 80 min at 30°C. The flow rate was 1 mL/min and fractions were collected each minute. The profile was monitored at 210 nm. (b) SDS-polyacrylamide gel electrophoresis of combined fractions from the separation in part 2a. Each lane is identified by the time of elution (min) of the peak material analysed. The migration of type I collagen components is indicated for the standard lanes (s), which contain 1.5 μg of hoki skin type I collagen (HS-pep).

time as temperature increased from 20°C to 35°C, but there was no change in the resolution of the peaks.

The effect of changing the flow rate was examined, while maintaining a constant gradient (rate of change of sodium ion concentration per mL) over the range 0.5 mL/min to 1.5 mL/min. The retention volumes, k and resolution (Rs) data calculated from the chromatograms shown in Figure 5 indicated that flow rate had no significant effect on the resolution of the collagen chains.

A major advancement in ion exchange packings occurred with the development of narrow diameter range monobeads sold as fast protein

Table 1. Selected groups of amino acids (mol %) in peaks collected from the chromatography of hoki type I skin collagen as shown in Figure 2a. Fraction labels relate to the peak labels on the chromatogram

	Collected fractions (mol %)					
Amino acid group	α_1	β_1	α_3	β_3	α_2	β_2
Imino acids Pro and Hyp	15.9	15.71	15.47	14.7	15.16	15.38
Thr and Ser	7.79	7.86	8.93	8.86	8.43	8.13
Acidic amino acids-Asp and Glu	14.06	14.32	14.59	15.03	13.06	13.51
Basic amino acids-Lys, Arg, His, Hly	9.44	9.39	9.64	9.67	10.08	9.82
Hydrophobic and aromatic- Tyr, Phe, Val, Leu, Ile, Met	6.17	6.29	7.36	7.72	7.61	7.06
Gly, Pro, Ala	55.87	55.62	53.3	52.75	54.97	55.4

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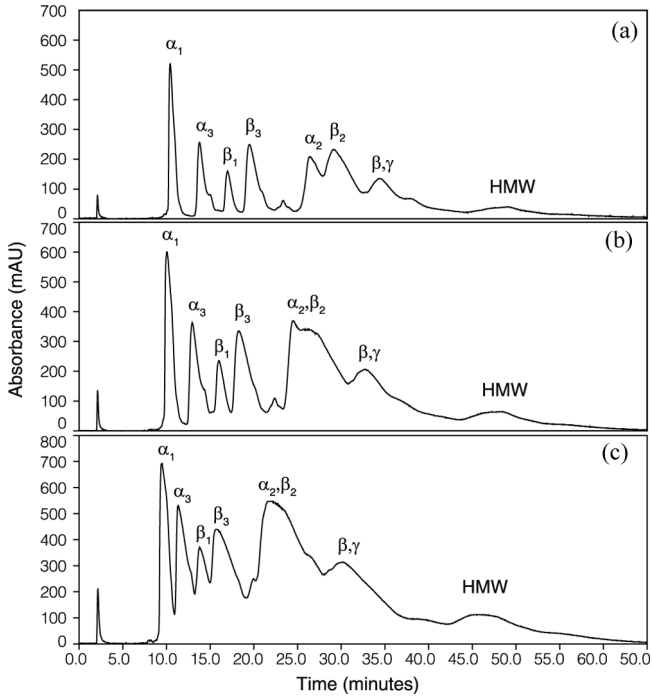

Figure 3. Elution profiles of heat denatured hoki skin type I collagen (HS-ASC) showing a comparison of the amount of collagen loaded onto a ProPac SCX column. The amounts loaded were (a) 0.36 mg, (b) 0.72 mg, and (c) 1.4 mg of sample. All samples were chromatographed at 30°C and 1 mL/min. The solvent was 50 mM sodium acetate-acetic acid at pH 5.0 and samples were eluted with a gradient program increasing the NaCl concentration from 0 to 0.2 M (5 min), 0.2 to 0.28 M (11 min), and 0.28 to 0.4 M (35 min). Profiles were monitored at 215 nm.

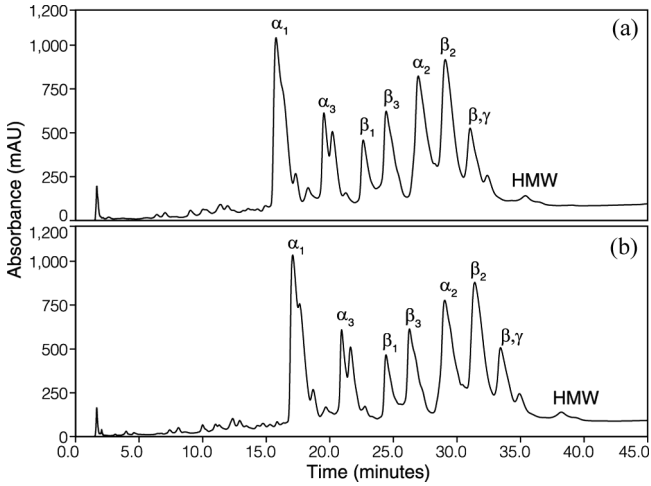

Figure 4. Profiles of heat denatured hoki skin type I collagen (HS-pep) chromatographed at (a) 20°C and (b) 35°C. Collagen (260 μg) was eluted using 1 mM sodium acetate-acetic acid at pH 4.4 with a linear gradient from 0.2 to 0.6 M NaCl over 50 min at 1 mL/min. The profile was monitored at 210 nm.

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liquid chromatography (FPLC™) in the early 1980 s. Bergstrom and his colleagues[16] recommended a gradient of 21.5 mM/ml ion concentration change with FPLC packings. A study was carried out on the ProPac SCX-10 packing with hoki collagen using 0.01 M acetic acid (pH 3.3) and changes in sodium ion concentration of 40 mM/mL, 20 mM/mL, and 10 mM/mL. The results (Figure 6) indicated that a gradient change of 20 mM/mL gave the optimised separation for hoki collagen at this pH, which is in agreement with the gradient change recommended by Bergstrom et al.[16] for FPLC packings.

Table 2. Sample retention on the column at different temperatures

Peak identification	Capacity factors (k') as measured at the different temperatures			
	20°C	25°C	30°C	35°C
α_1	8.78	9.04	9.33	9.56
α_3	11.11	11.38	11.67	11.93
β_1	13.00	13.33	13.67	14.11
β_3	14.09	14.49	14.82	15.22
α_2	15.64	16.09	16.44	16.89
β_2	16.89	17.44	17.89	18.33
γ	18.11	18.60	19.09	19.56

$k' = t_1 - t_0/t_0$ where t_0 is the column void time and t_1 is peak retention time.

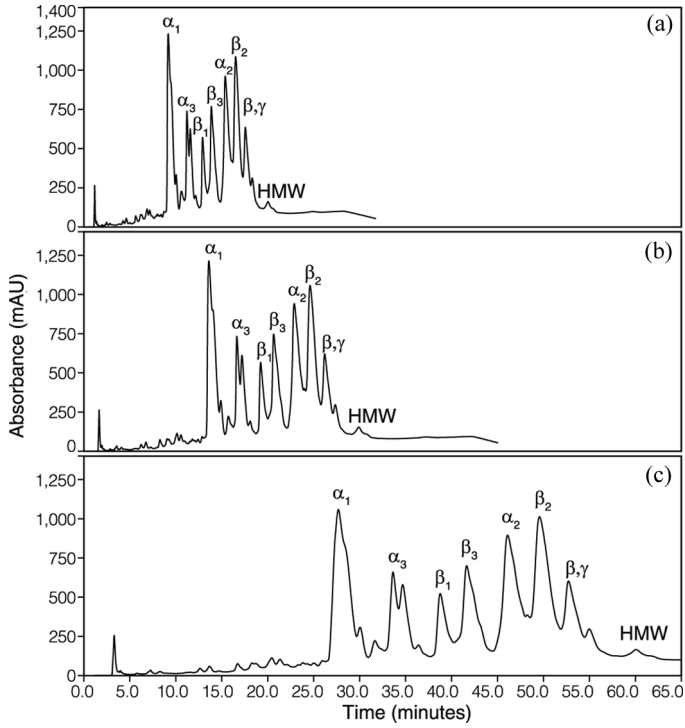

Figure 5. Profiles of heat denatured hoki skin type I collagen (HS-pep) chromatographed at (a) 1.5 mL/min, (b) 1 mL/min, and (c) 0.5 mL/min. Collagen (260 μg) was eluted using 1 mM sodium acetate-acetic acid at pH 4.4 with a linear gradient from 0.2 to 0.6 M NaCl. A 40 mL gradient volume with a 10 mM change in sodium ion concentration per mL was used for all profiles. Elution was monitored at 210 nm. The column temperature was 30°C.

When we determined that pH 4.4 gave better separation of hoki collagen, a more detailed study of the effect of gradient change on resolution was carried out with 1 mM sodium acetate-acetic acid (pH 4.4) (Figure 7). The most difficult peak to resolve completely was the α_2 chain, which either partially or completely coeluted with a β component under most conditions studied. When the effect of the gradient slope was examined over the range 2–10 mM/mL sodium ion concentration, it was found that the α_2 peak was the only component that moved significantly. This is reflected in the Rs data in Table 3. Therefore, gradient manipulation may help resolve a particularly difficult part of the separation. However, the practical disadvantages of increased running time and larger collection volumes for individual peaks means that a gradient change of 10–20 mM/mL of eluting ion is recommended for investigating solvent conditions.

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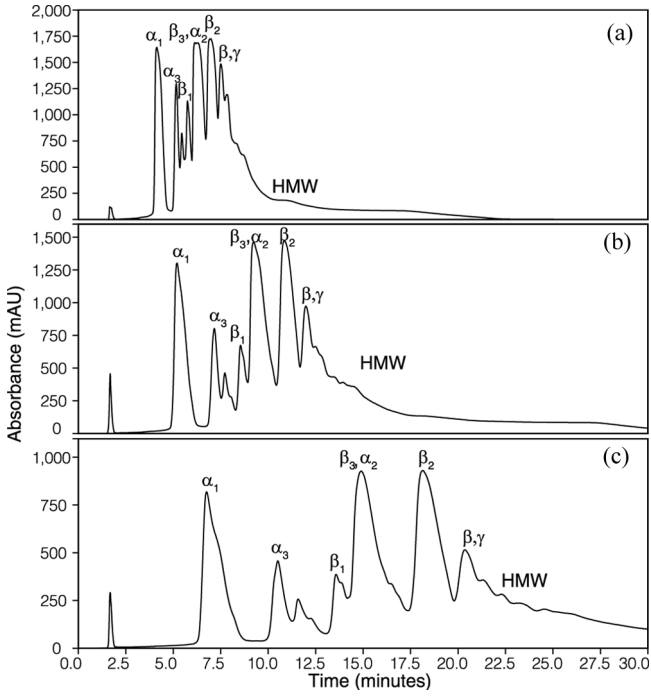

Figure 6. Profiles of heat denatured hoki skin type I collagen (HS-ASC) chromatographed with different gradient steepness. The rate of change of sodium ion concentration was (a) 40 mM/mL, (b) 20 mM/mL, and (c) 10 mM/mL, respectively. Collagen (360 μg) was separated with 0.01 M acetic acid, pH 3.3, and the samples were eluted with a linear gradient from 0.4 to 0.8 M NaCl over (a) 10 min, (b) 20 min, and (c) 40 min at 30°C and 1 mL/min. Profiles were monitored at 210 nm.

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The lack of amino acids in collagen that absorb at 280 nm necessitates UV detection at <220 nm and restricts the choice of solvents when UV absorbance is to be used to detect this protein. Solvents used in this study were 0.01 M acetic acid (pH 3.3), 1 mM sodium acetate-acetic acid (pH 4.0), 50 mM sodium acetate-acetic acid (pH 5.0), and 20 mM phosphate buffer at pH 6.0, to examine the effect on resolution of the hoki collagen over the pH range 3–6 (Figure 8). The gradients were initiated at increasing concentrations of NaCl to account for the increased binding as the pH was lowered. Long runs initiated with no NaCl in the column did not change the resolution patterns from those indicated in Figure 8. The peak most influenced by the pH was the α_2 chain, which coeluted with the β_2 and γ material at pH 6, eluted as a shoulder of the β_2 component at pH 5.0, and was only partially resolved

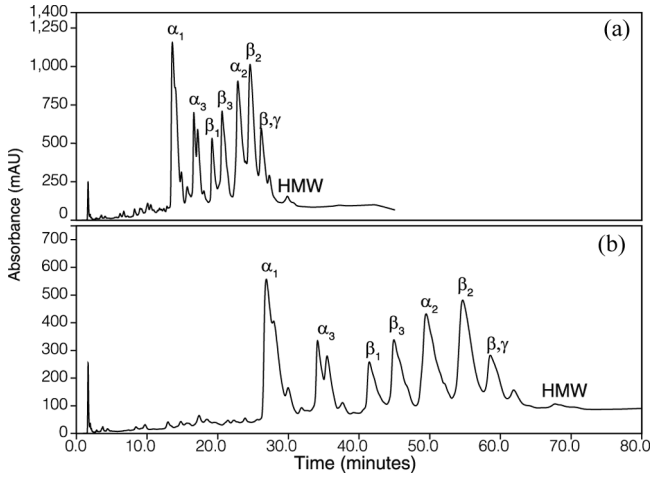

Figure 7. Profiles of heat denatured hoki skin type I collagen (HS-pep) chromatographed with different gradient steepness. The rate of change of sodium ion concentration was (a) 10 mM/mL and (b) 4 mM/mL, respectively. Collagen (260 μg) was separated with 1 mM sodium acetate-acetic acid, pH 4.4, and the samples were eluted with a linear gradient from 0.4 to 0.6 M NaCl over (a) 40 min and (b) 100 min at 30°C and 1 mL/min. Profiles were monitored at 210 nm.

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from the β_3 component at pH 4.0. Therefore, a pH of 4.4 was selected as an optimal pH to elute all the fractions of the hoki type I collagen. This separation is shown in Figure 2a. The pH of fractions collected throughout the gradient was measured to confirm that the concentration of 1 mM sodium acetate-acetic acid was sufficient to maintain the pH with changing protein and salt concentrations. The low buffer concentration reduced the background absorbance and increased detection sensitivity.

Table 3. Resolution (Rs) between peaks

Peaks resolved	Eluting ion concentration				
	10 mM/mL	8 mM/mL	6 mM/mL	4 mM/mL	2 mM/mL
α_1–α_3	2.94	2.60	2.51	3.06	3.03
α_3–β_1	2.93	2.66	2.50	3.31	3.34
β_1–β_3	1.20	1.09	1.0	1.14	1.02
β_3–α_2	1.40	1.35	1.11	1.16	0.98
α_2–β_2	0.98	1.05	1.10	1.30	1.44
β_2–γ	0.90	1.0	0.97	0.95	0.90

$Rs = 2x\ (T_{R1} - T_{R2})/(w_1 + w_2)$ [15].

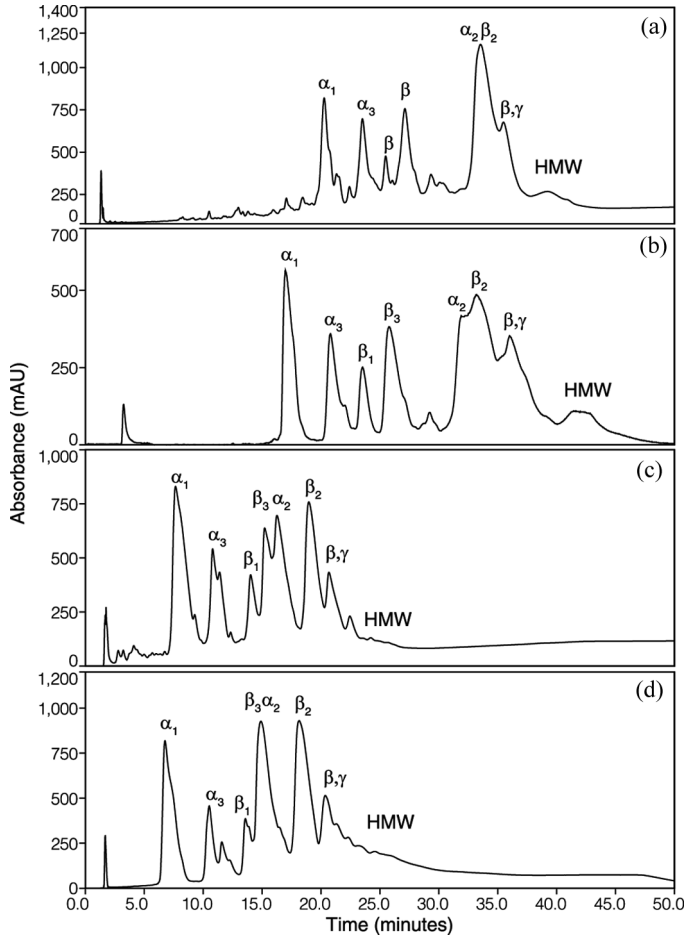

Figure 8. Profiles of heat denatured hoki skin type I collagen chromatographed with different solvents at different pHs. The solvents used were (a) 20 mM phosphate buffer at pH 6.0, (b) 50 mM sodium acetate-acetic acid at pH 5.0, (c) 1 mM sodium acetate-acetic acid pH 4.0, and (d) 0.01 M acetic acid at pH 3.3. The gradient change of sodium ion concentration was (a) 8.3 mM/mL, (b) 12 mM/mL, and (c and d) 10 mM/mL, respectively. The gradients were initiated at (a) 0 M, (b and c) 0.2 M, and (d) 0.4 M NaCl, respectively. Profiles were monitored at 210 nm except (b), which was monitored at 215 nm. Samples in (a), (b), and (d) were extract HS-ASC and sample (c) was HS-pep. All samples were analysed at 30°C.

Urea is commonly added in low pressure ion exchange chromatography of collagen chains. This is thought to prevent aggregation and improve separation. It was found that the addition of urea to the solvent caused

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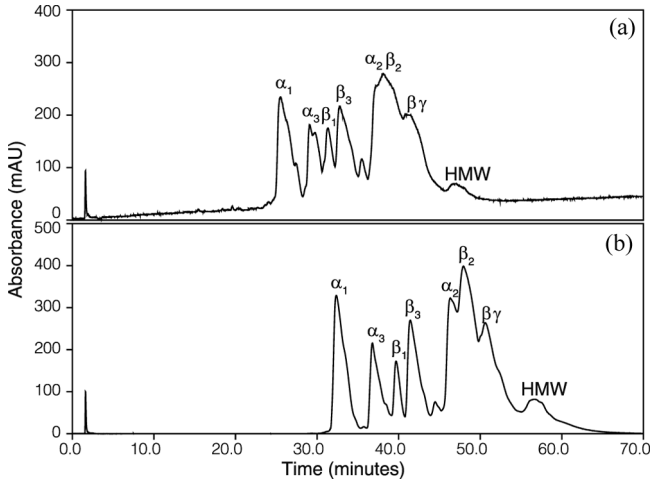

Figure 9. Elution profiles of heat denatured hoki skin type I collagen showing the effect of urea in the solvent. In sample (a) 2 M urea was added to the solvent, 20 mM sodium acetate-acetic acid (pH 4.8), and sample (b) was eluted with 20 mM sodium acetate-acetic acid (pH 4.8) only. Collagen (360 μg) was eluted using a linear gradient from 0 to 0.5 M NaCl over 80 min at 1 mL/min. Sample (a) was HS-pep and (b) was HS-ASC. Profiles were monitored at 215 nm.

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the peaks to elute earlier (Figure 9). However, even when the gradient was altered to retain the samples on the column to improve the analysis, the inclusion of urea (either 2 M or 4 M) was detrimental to the resolution of the hoki collagen components on the Dionex ProPac SCX packing.

DISCUSSION

In all HPLC of collagen α chains reported in the literature (whether reverse phase, gel permeation with secondary effects or ion exchange), the α_2 chain always elutes after the α_1(I) and α_1(III) chains and often in combination with the β, γ, and HMW fractions.[7–11]

In ion exchange chromatography of collagen chains, separation is influenced by the way the charge of the chains is presented to the packing, the strength of the ionic interaction as determined by the pKas of the individual amino acid residues and the pH of the solvent.

Collagen is an amphoteric ionic structure attaining the highest degree of charge, both positive and negative, in the iso-electric pH range.[17] It is known that substances usually begin to dissociate from ion exchangers about 0.5 pH units from their iso-electric points (IEP) at ionic strength 0.1. Acid soluble collagen has an IEP of pH 7.0 at low (0.02 N) ionic

strength.[17] With increasing ionic strength the uptake of anions by the protein decreases the IEP such that in 0.1 M solvent the IEP of acid extracted collagen from hide was pH 5. Similarly, acid soluble marine collagen from cod skin indicated an IEP of pH 5 when analysed in 0.1 M citrate buffer.[18] The optimised separation presented in this work uses a NaCl concentration from 0.2 to 0.52 M (Figure 2) at pH 4.4. As the ionic concentration increases during the gradient elution process, the charge on the polar R groups may be influenced by the uptake of anions by the collagen chains. However, the lowest pH value of the IEP recorded for gelatin after months of liming (which hydrolyses the amide groups) was 4.5,[17] indicating limits to the change in IEP that can result from anion uptake. Therefore, the chromatography conditions at pH 4.4 optimize both the charge on the protein, bringing it near the IEP in the ionic strength range used, and the dissociation properties of amphoteric molecules from ion exchangers (about 0.5 pH units from their IEP at 0.1 M ionic strength).

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The content of polar amino acids is indicated for the three alpha chains in Table 1. The order of elution reflects the content of basic amino acids as would be expected for a cation exchange separation; the α_2 chain containing the highest mol % of basic amino acids is the most strongly bound to the column. Similarly, the lower content of total acidic amino acids in the α_2 chain coincides with its expected elution position when compared to the Glu and Asp contents of the α_1 and α_3 chains. The mol % differences in the polar amino acid content of the three α chains are small, but this column is sufficiently selective to enable the separation of all the collagen components under the optimized conditions.

In mammalian type I collagen the length of the telopeptides differs between the α_1(I) and α_2(I) chains—containing a total of 42 residues for the combined telopeptides of α_1(I) compared with 26 residues for α_2(I).[19] Whether these differences influence the behaviour of the α chains in chromatography, or are reflected across marine species, is unknown.

The ProPac SCX-10 column packing (10 μm particles) is a rigid, non-porous pellicular resin with a hydrophilic layer to prevent hydrophobic interaction with proteins. The loading capacity for the 4×250 mm column is quoted as >100 mg of protein without loss of resolution depending on the specific protein. It is not surprising that the loading capacity for collagen is significantly lower than 100 mg when the size and shape of collagen components are considered. The characteristic sequence containing glycine at each third residue in the helical portion of the α chains and the high content of imino acids must influence the shape of the denatured collagen components in solution. The restriction in rotation of bonds adjacent to a pyrrolidine ring, along with the double bond character of the peptide link, is proposed to determine the relative positions of six consecutive bonds along a peptide chain.[5] That is, even

in denatured form the individual chains contain drivers for rigidity in the secondary structure. This is supported by Gustavson, who proposed that "the architecture of the molecule influences the function and properties of the positively and negatively charged centers of the dipolar structure in several ways. Thus, the charged sites of the collagen chains do not have the freedom of action found with ions of soluble amphoteric electrolytes."[17] This implies that the denatured chains will present to the column packing with greater surface area than a globular protein of equivalent molecular weight. It is proposed that the high molecular weight and rigid structure of the collagen chains influence the loading capacity of this column. The optimal loading of the hoki collagen was found to be in the order of 0.3–0.4 mg. Flow rate was shown to have no influence on the resolution of the collagen components on this column.

In reversed phase HPLC of denatured collagens, the molecular size difference between α, β and γ components makes a relatively minor contribution to separation.[6] The larger β and γ components were usually the last to elute from these columns, indicating that these larger fragments take longer to move through the packing than the smaller α chains. In this work, the γ components and higher molecular weight aggregates >300 kDa (when present) were the last to elute from the ion exchange packing. However, two β components were resolved prior to the elution of the α_2 chain, indicating that while size ≥300 kDa affected the time of elution from the packing, fragments ≤200 kDa were resolved independent of size.

The increased retention times with increased column temperature observed with this column were unexpected since the packing is modified to mask potential hydrophobic interactions. It is known that hydrophobic interactions are strengthened by increasing temperature. Although present in low concentrations, the order of elution does reflect the content of amino acids with hydrophobic character (Table 1), being 6.17, 7.36, and 7.61 mol % for the α_1, α_3, and α_2 chains, respectively. The charged and hydrophobic groups are found in clusters along the collagen molecule.[21] It is proposed that hydrophobic interactions within the collagen chains have the potential to influence the presentation of the charged amino acids to the packing material and thus influence retention. This is supported by the observation that the addition of urea in this system caused a reduction in both retention time and resolving capability. Urea is traditionally used in low pressure ion exchange chromatography of collagen to prevent aggregation since it has the potential to disrupt hydrogen bonded water structure and influence the solubility of non-polar residues. Urea is expected to prevent hydrophobic interactions within the chains and it appears to concurrently influence the presentation of the charged groups to the packing surface. The result is a negative effect on

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the retention and resolution of the hoki collagen chains in the presence of this additive.

CONCLUSION

The separation of collagen chains for further analysis and characterization in the discovery and study of disease has proved challenging. Hoki collagen, similar to other collagens from species living in low temperature environments, is low in imino acid content compared with mammalian collagens. This may influence the flexibility of the chains in solution and the presentation of its molecular components to the ion exchange packing and thus facilitate resolution. This investigation using a marine collagen has provided further insight into how collagen chains are influenced by changing parameters in ion exchange chromatography. While flow rate was shown not to influence resolution, column loading was limited by molecular architecture. An increase in retention time with increasing temperature was attributed to enhanced intrachain hydrophobic interaction, influencing the presentation of charged groups to the packing surface. This was supported by decreased retention and resolution in the presence of urea. While the α_1 and α_3 chains were readily separated, the α_2 chain was the most challenging component to resolve from β material, eluting closely associated with either β_3 or β_2 depending on the conditions. The α_2 chain was also the most mobile component and was the most influenced by changing parameters, responding to both gradient change and pH. The slope of the eluting ion change was shown to cause the α_2 chain to shift in relation to neighboring peaks, as well as give more space between all partially resolved peaks. The most effective parameter for change in resolution of the hoki collagen chains was the pH. These results point to the potential for combined pH and salt gradients to enable resolution for difficult collagen separations.

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REFERENCES

1. Hearn, M.T.W. High-performance liquid chromatography and its application to protein chemistry. Adv. Chromatogr. **1982**, *20*, 1–82.

2. Hancock, W.S.; Sparrow, J.T. The separation of proteins by reversed-phase high-performance liquid chromatography. HPLC. **1983**, *3*, 49–73.
3. Deyl, Z.; Miksik, I. Advanced separation method for collagen parent α-chains, their polymers and fragments. J. Chromatogr. B. Biomed. Appl. **2000**, *739*, 3–31.
4. Deyl, Z.; Miksik, I.; Eckhardt, A. Preparative procedures and purity assessment of collagen proteins. J. Chromatogr. B. Biomed. Appl. **2003**, *790*, 245–275.
5. Fallon, A.; Lewis, R.V.; Gibson, K.D. Separation of the major species of interstitial collagen by reverse-phase high-performance liquid chromatography. Anal. Biochem. **1981**, *110*, 318–322.
6. Van Der Rest, M.; Bennett, H.P.J.; Solomon, S.; Glorieux, F.H. Separation of collagen cyanogen bromide-derived peptides by reversed-phase high-performance liquid chromatography. Biochem. J. **1980**, *191*, 253–256.
7. Smolenski, K.A.; Fallon, A.; Light, N. Investigation of the parameters for reversed-phase high-performance liquid-chromatography of collagen Type-I and Type-III. J. Chromatogr. **1984**, *287*, 29–44.
8. Skinner, S.J.M.; Grego, B.; Hearn, M.T.W.; Liggins, G. The Separation of collagen alpha chains by reversed-phase high-performance liquid chromatography. Comparison of column alkyl stationary phases and temperature effects. J. Chromatogr. **1984**, *308*, 111–119.
9. Van Der Rest, M.; Fietzek, P.P. A Comprehensive approach to the study of collagen primary structure based on high-performance liquid chromatography. Eur. J. Biochem. **1982**, *125*, 491–496.
10. Macek, K.; Deyl, Z.; Coupek, J.; Sanitrak, J. Separation of collagen Types I and III by high-performance column liquid chromatography. J. Chromatogr. **1981**, *222*, 284–290.
11. Bateman, J.F.; Mascara, T.; Chan, D.; Cole, W.G. Rapid fractionation of collagen chains and peptides by high-performance liquid chromatography. Anal. Biochem. **1986**, *154*, 338–344.
12. Miller, E.J.; Furuto, D.K.; Narkates, A.J. Quantitation of Type I, III and V collagens in human tissue samples by high-performance liquid chromatography of selected cyanogen bromide peptides. Anal. Biochem. **1991**, *196*, 54–60.
13. Gornall, A.; Bardawill; David, J. Determination of serum proteins by means of the biuret reaction. Biol. Chem. **1949**, *177*, 751–766.
14. Ramshaw, J.A.M.; Werkmeister, J.A.; Bremner, H.A. Characterization of Type I collagen from the skin of blue grenadier (*Macruronus novaezelandiae*). Arch. Biochem. Biophys. **1988**, *267*, 497–502.
15. Purnell, J. Liquid chromatography theory. J. Chem. Soc.. **1960**, 1268–1274.
16. Bergstrom, J.; Soderberg, L.; Wahlstrom, L.; Muller, R.; Domicelj, A.; Hagstom, G.; Stalberg, R.; Kallman, I.; Hansson, K. Recommendations for obtaining optimal separations on monobead ion exchangers. Protides Biolog. Fluids. **1983**, *30*, 641–646.
17. Gustavson, K. *The Chemistry and Reactivity of Collagen*; Academic Press Inc.: New York, 1956; 93–103.
18. Lampson, V.; Tytell, A.A. A simple method for estimating isoelectric points. Anal. Biochem. **1965**, *11*, 374–377.

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19. Veis, A.; Payne, K. in *Collagen*; Nimni, M., Ed.; CRC Press, Inc: Florida, 1988, 113–137.
20. Piez, K.A.; Gross, J. The amino acid composition of some fish collagens: The relation between composition and structure. J. Biol. Chem. **1960**, *235*, 995–997.
21. Fietzek, V.; Kuhn, K. The primary structure of collagen. Int. Rev. Connect. Tissue Res. **1976**, *7*, 1–60.

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