- Protonated amino groups at the N-terminus, in lysine side-chains, the guanidino moiety of arginine, and the imidazole ring of histidine contribute positive charges.
- Deprotonated carboxyl groups at the C-terminus and in side chains of glutamic and aspartic acid residues produce negative charges.
- Amidated carboxyls, the N-terminal pyroglutamate (a cyclic amide) of neurotensin, cysteine sulfhydryls (under reducing conditions), and the phenolic hydroxyl of tyrosine are not sufficiently acidic or basic to carry charge in this pH range.

Included with the pKa value in each flag are two symbols for charge states (+, -, or 0). The upper symbol indicates the predominant charge at pH values above pKa and the lower symbol the predominant charge at pH values below pKa. At pH differing from pKa by one or more, the group will carry a full charge of the sign indicated. At pH equal to pKa the group should be counted as 1/2 charge. The sum of contributions from all charged groups in the structure at each pH gives the approximate net charge on the peptide, shown in the table for four typical pH values.

net charge			
pH 2	pH 4.1	pH 6	pH 8
+1	+1	+1	+0.5
+3	+2.5	+2	+2
+3	+2	+0.5	0
+2	+2	+2	+2
+3	+2	+1	+1
+4	+3	+1	0

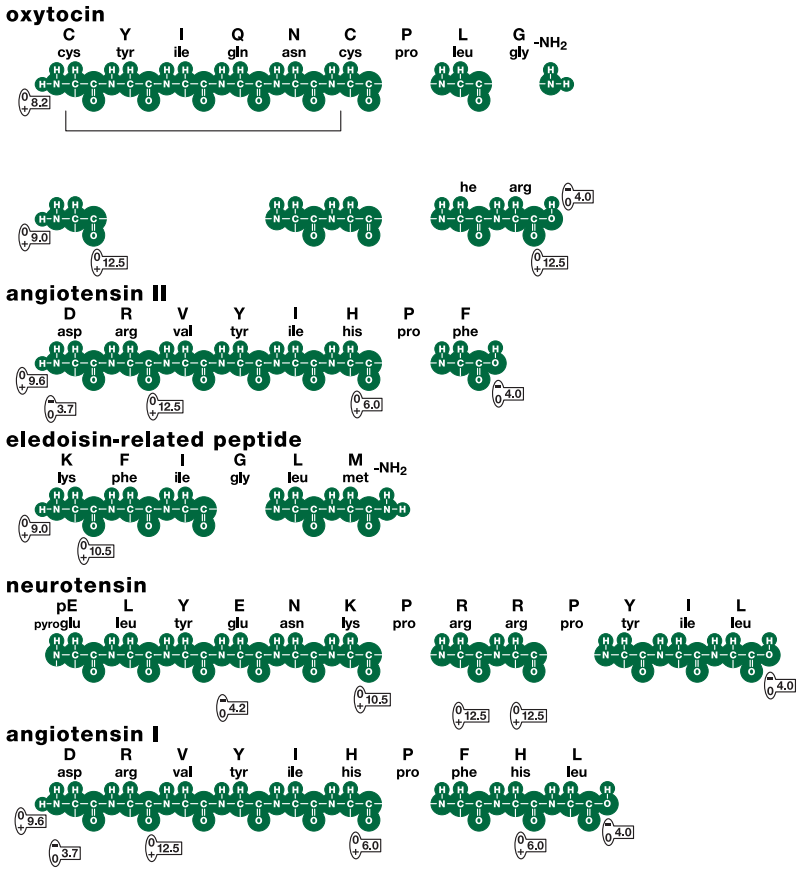

Figure 1. Calculating the net charge on a peptide as a function of pH.
Flags adjacent to ionizable functional groups in the structure indicate pKa values together with the predominant charge state of that functional group for pH values above the pKa (upper symbol) and for pH values below the pKa (lower symbol). For pH = pKa, charge is 1/2.

Results and Conclusions

Separations of the six peptides carried out at pH 2 and pH 4.1 are shown in Figure 2. In each case the sample was loaded at pH 2. For the pH 4.1 separations, a 15 minute rinse at pH 4.1 was followed by a gradient of salt concentration.

Figure 2a shows the separation in buffers containing 50% ACN with a salt gradient from 0 to 100mM $NaClO_4$ over 50 minutes. Significant observations are:

- Oxytocin, with a net charge of +1 is retarded slightly but not retained on this column.
- Three peptides all carrying a net charge of +2 are retained and baseline separated. Hydrophilic effects are responsible for the resolution of these peaks.
- Conversely, two peptides carrying significantly different net charges (bradykinin, +2.5; angiotensin I, +3) elute together. Hydrophilic effects are powerful enough to overcome the charge difference.

The separation of Figure 2b is similar. However, a shallower salt gradient, from 0 to 50mM $NaClO_4$ over 50 minutes, was able to partially resolve bradykinin and angiotensin I.

Figure 2c shows the separation run with the same gradient as 2a, but with 25% ACN instead of 50% ACN. Organic modifiers are known to strengthen ionic interactions resulting in complex effect on peptide retention (Ref. 1). As expected for small peptides, all components of the test mixture were less strongly retained in the lower ACN concentration due to weaker ionic interaction with the stationary phase. In fact, most of the peaks emerged during the period of change to pH 4.1, before the onset of the salt gradient. Interestingly, bradykinin and angiotensin I are completely resolved under these conditions, possibly as a result of hydrophilic effects, or possibly because separation has occurred during the period before complete pH equilibration.

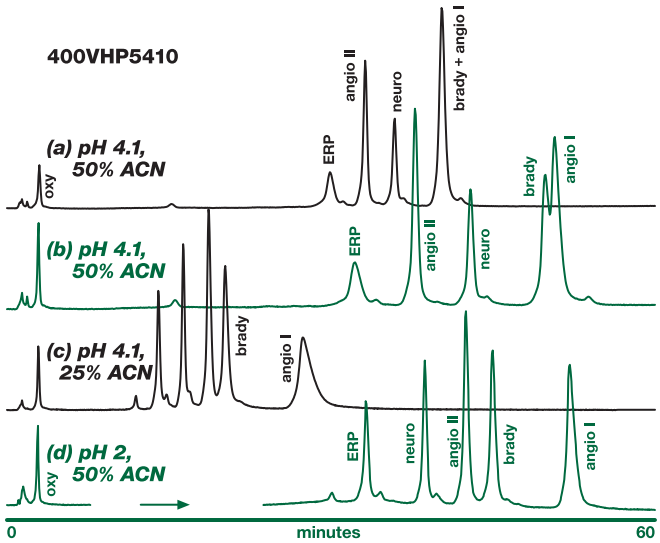

Figure 2. Separation of six peptides by cation exchange chromatography. Column: Vydac 400VHP5410 (4.6mmID x 100mmL). Flow: 0.7 mL/min. Detection: 220 nm. Mobile phase: (a) A = 20mM TEAP, pH2, 50% ACN. B = 20mM TEAP, pH4.1, 50% ACN. C = 100mM $NaClO_4$ in B. Gradient: 100%A to 100%B in 1 minute. Hold 100%B for 15 minutes. Then linear 100%B to 100%C over 50 minutes. (b) Same as (a), except linear 100%B to 50%B:50%C over 50 minutes. (c) Same as (a), except 25% ACN. Gradient: 100%A to 100%B over 15 minutes. Then linear 100%B to 100%C over 50 minutes. (d) A = 20mM TEAP, pH2, 50% ACN. B = 100mM $NaClO_4$ in A. Gradient: 100% A to 100%B over 50 minutes. Chromatogram (d) displaced 16 minutes at break to compensate for absence of pH change period.

Finally, Figure 2d shows the separation run at pH 2 throughout with the higher ACN concentration and same salt gradient as 2a. The baseline break indicates the point at which elution times have been shifted 16 minutes to compensate for the fact that the salt gradient was commenced immediately (without the initial hold for pH shift). It can be seen that at pH 2 and 50% ACN good retention and complete resolution were obtained for all peaks. However, the elution of angiotensin II and neurotensin are reversed, a phenomenon that cannot be explained by charge effects.

The order of elution for neurotensin, angiotensin II, and bradykinin in Figure 2d, at a pH where all three carry similar net charge, is the best evidence that hydrophilic effects are responsible for their separation. It is the reverse of the order of elution seen on a reversed-phase column where retention is due to hydrophobic interactions (Ref. 2).

Summary

- Net charges on peptides can be manipulated by changing pH.
- An organic-solvent modifier increases ionic retention and hydrophilic effects.
- Hydrophilic effects play a role in peptide separation by cation exchange.
- Optimizing a peptide separation may require manipulating first pH and then solvent concentration.

References

1. Vydac Application Note #9808.
2. Vydac Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC (Second Edition, 1995), Figure 9, page 10.

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Cat.No.	Description
400VHP5410	Column, Cation-Exchange Sulfonic Acid, 900Å, 5µm, 4.6mm ID x 100mm L

Other column sizes are available for analytical and preparative applications.