Ion-Exchange Chromatography: Basic Principles and Application to the Partial Purification of Soluble Mammalian Prolyl Oligopeptidase

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Abstract

Ion-Exchange Chromatography (IEC) allows for the separation of ionizable molecules on the basis of differences in charge properties. Its large sample-handling capacity, broad applicability (particularly to proteins and enzymes), moderate cost, powerful resolving ability, and ease of scale-up and automation have led to it becoming one of the most versatile and widely used of all liquid chromatography (LC) techniques. In this chapter, we review the basic principles of IEC, as well as the broader criteria for selecting IEC conditions. By way of further illustration, we outline protocols necessary to partially purify a serine peptidase from bovine whole brain cytosolic fraction, covering crude tissue extract preparation through to partial purification of the target enzyme using anion-exchange chromatography. Protocols for assaying total protein and enzyme activity in both pre- and post-IEC fractions are also described. The target serine peptidase, prolyl oligopeptidase (POP, EC3.4.21.26), is an 80 kDa enzyme with endopeptidase activity towards peptide substrates of ≤30 amino acids. POP is a ubiquitous post-proline cleaving enzyme with particularly high expression levels in the mammalian brain, where it participates in the metabolism of neuroactive peptides and peptide-like hormones (e.g. thyroliberin, gonadotropin-releasing hormone).

Key Words: Liquid Chromatography, Anion-Exchange, Cation-Exchange, Matrix, pH, Ionic Strength, DEAE-Sepharose® Fast Flow, Prolyl Oligopeptidase, Bovine Brain

1. Introduction

Bioseparation involves resolution of the components in complex mixtures encountered in biological and biochemical systems, thereby enabling scientists to determine both the identity and concentration of each component and, if necessary, to isolate a desired component from other contaminating molecules for further analysis or application. Bioseparation processes are frequently dominated by liquid chromatography (LC) steps. Resolution of mixtures by LC is based on the principle that, under a given set of conditions, individual solutes dissolved in a *mobile phase* will differentially interact with a chemically modified *stationary phase* as a function of differences in individual solute distribution coefficients (K). In this way, LC exploits inherent differences between biomolecules (e.g. molecular size, hydrophobicity, binding specificity, charge) in order to achieve their separation from one another.

1.1 Basic Principles of Ion-Exchange Chromatography

With its origins dating back to the 1940s, ion-exchange chromatography (IEC) was designed specifically for the separation of differentially charged or ionizable molecules ***(1, 2)***. Both chemists and biochemists have routinely employed this technique for the purification of proteins ***(3, 4)***, enzymes ***(4, 5)***, antibodies ***(3, 7)***, peptides ***(8)***, amino acids and nucleic acids ***(9, 10)***, as well as simpler carbohydrates ***(11)*** and organic compounds ***(12)***. Its large sample-handling capacity, broad applicability (including high performance and high-throughput application formats), moderate cost, powerful resolving ability, and ease of scale-up and automation have led to it becoming one of the most versatile and widely used of all LC techniques. Like other forms of column-based LC (e.g. gel-permeation, affinity, hydrophobic interaction etc.), this technique comprises both mobile and stationary phases, the former typically an aqueous buffer system into which the mixture to be resolved is introduced, and the latter usually an inert organic matrix chemically derivatized with ionizable functional groups that carry a displaceable oppositely charged counterion. These counterions exist in a state of equilibrium between the mobile and stationary phases, giving rise to two possible IEC formats, namely anion- and cation-exchange (*see* **Fig. 1**). Exchangeable matrix counterions may include protons (H^+), hydroxide groups (OH^-), single charged monoatomic ions (Na^+, K^+, Cl^-), double charged monoatomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}), as well as organic bases (NR_2H^+) and acids (COO^-).

The pH-dependent ionization of electrolyte groups (weak acids or bases) can impart a net positive or negative charge on biomolecules, subsequently enabling their separation from one

another via IEC. This can be explained by taking as an example the separation of a mixture of proteins. During IEC, the mobile phase pH will determine the net charge on both the matrix functional group and on individual proteins within the sample mixture. The polyampholyte nature of proteins mean they can carry both positive and negative charges, the former largely attributable to the ionization of lysine and arginine side chains, and the latter to aspartate and glutamate side chain ionizations. As a general rule, a protein will have a net negative charge above its isoelectric point or p*I* (i.e. pH at which a protein has zero net charge), and vice-versa. Naturally, different proteins may have different p*I* values, and therefore a different net charge at any given pH. A mobile phase pH can therefore be selected to ensure that the net charge on a protein of interest within a mixture is opposite to that of the matrix functional group, ensuring that it will displace the functional group counterion and bind the matrix (adsorption). Conversely, oppositely charged 'contaminant' proteins will not be retained.

Bound protein analytes can be eluted (desorption) in one of two possible ways: (i) pH; (ii) ionic strength. Changing the mobile phase pH alters the net charge of the bound protein, and thus its matrix binding capacity. More commonly, increasing the concentration of a similarly charged species within the mobile phase can compete with and ultimately displace the bound ionic species. During anion-exchange chromatography for example, negatively charged protein analytes can be competitively displaced by the addition of negatively charged chloride ions (e.g. from sodium chloride). By gradually increasing the salt concentration in the mobile phase, the affinity of interaction between the salt ions and the functional groups will eventually exceed that which exists between the protein charges and the functional groups, resulting in protein displacement and elution.

1.2 Criteria for Selection of IEC Conditions

In the following sub-sections (1.2.1 to 1.2.4), the broader criteria for selecting IEC conditions is discussed, thereby allowing one to better comprehend and adapt the described protocols ***(13)***. The ensuing materials and methods sections (sections 2. & 3.) outline the protocols necessary to partially purify a serine peptidase from bovine whole brain soluble fraction. Specifically, we describe the preparation of a crude tissue extract using homogenization, centrifugation and ammonium sulphate precipitation, followed by partial purification using anion-exchange LC. To allow one to accurately monitor purification efficiency, protocols for assaying total protein and enzyme activity in both pre- and post-IEC fractions are also described. Our focus for these protocols is prolyl oligopeptidase (POP, prolyl endopeptidase,

EC3.4.21.26), an 80 kDa serine peptidase with endopeptidase activity towards peptide substrates of ≤30 amino acids. POP is a ubiquitous post-proline cleaving enzyme with particularly high expression levels in the mammalian brain, where it participates in the metabolism of neuroactive peptides and peptide-like hormones (e.g. thyroliberin, gonadotropin-releasing hormone, substance P, Arg-vasopressin). Several studies indicate putative roles for POP in regulation of the central nervous system at both the physiological (memory, learning, mood) and pathological (Alzheimer's, Huntington's and Parkinson's diseases) levels ***(14, 15)***.

1.2.1 Selection of ion-exchange matrix

The choice of a suitable ion-exchange matrix is probably the single most important aspect of any ion-exchange protocol and is based on various factors, which include: (i) desired ion-exchanger charge/strength; (ii) linear flowrate/sample volume; (iii) sample properties. As mentioned above, ion-exchange functional groups fall into two charge categories. Positively charged diethylaminoethyl (DEAE) and quarternary ammonium (Q) functional groups for example, are routinely employed in anion-exchange chromatography, whilst negatively charged carboxymethyl (CM), sulphomethyl (S) and sulphopropyl (SP) groups are typical cation-exchangers. Both exchanger types can be further categorized as either "strong" or "weak". Strong ion-exchangers are fully ionized over a broad working pH range (i.e. show no loss or gain of charge with varying pH), whilst weak ion-exchangers are only partially ionized over a narrow pH range (i.e. charge can vary significantly with pH). Consequently, with strong ion-exchangers, individual proteins can adsorb to several exchanger sites, often necessitating harsh elution conditions (up to 1 *M* NaCl) that may compromise sample stability and resolution. Strong ion-exchangers are therefore often used for initial development and optimization of purification protocols (and for binding proteins with p*I* values lying towards the more extreme ends of the pH scale). By contrast, weak ion-exchangers are more flexible in terms of selectivity, and are a more common choice for the separation of proteins that retain their functionality over the pH 6-9 range, as well as for labile proteins that may require mild elution conditions (**Table 1** highlights a range of commercially available ion-exchanger resins categorized on the basis of charge and strength).

The size, porosity and binding capacity of resin particles are also important when selecting an ion-exchanger matrix. These resin properties are normally based on chromatographic column size and dimensions, in conjunction with sample volume and concentration. Commercially

available resins range in size from 10 to 400 microns. Larger particles are frequently used in initial protein purification stages that require fast elution rates and high capacity but low-to-intermediate resolution, whilst smaller particles are ideal for final purification stages requiring higher resolution. Moreover, commercial resins have binding capacities ranging from less than 2 mg/mL to more than 150 mg/mL.

Selection of the most suitable exchanger functional group for a purification will also be dictated by the target protein biochemical properties such as p*I* and pH stability. For example, if a protein has a low p*I* (<5.0), but is more stable at pH values above this, then an anion-exchanger should be chosen, and vice-versa. The purification of prolyl oligopeptidase by ion-exchange chromatography is a good example. With a p*I* in the region of 4.8 ***(16, 17)***, prolyl oligopeptidase could potentially adsorb to either a strong cation-exchanger or a weak-anion exchanger. However, with a pH optimum from pH 7.4-8.0, coupled with rapid destabilization of the enzyme below pH 5.0 (Dr. Oonagh Dowling - unpublished observations), the anion-exchange option is favored. To illustrate this, **Fig. 2** (and **Table 2**) demonstrates how anion-exchange chromatography using DEAE-Sepharose® Fast Flow (GE Healthcare) can be used for the initial-stage purification of prolyl oligopeptidase from bovine whole brain soluble tissue extract. Of relevance, the broad pH stability previously reported for the "serum" forms of POP and its *Z*-Pro-Prolinal-insensitive homolog (ZIP) ***(18, 19)*** has previously been exploited in a cation-exchange protocol to resolve one isoform from the other ***(20)***.

1.2.2 Selection of buffer conditions

In order to prevent any variation in matrix and protein net charge, maintenance of a constant mobile phase pH during IEC is essential to avoid pH fluctuations which can occur when both protein and exchanger counterions (particularly if these are H^+ or OH^- ions) are released into the mobile phase. A number of important factors dictate choice of the mobile phase buffer, which include: (i) buffer charge; (ii) buffer strength; (iii) buffer pH. The buffering ion should not interact with the ion-exchanger functional groups (i.e. positively charged buffers should be used in anion-exchange, and vice-versa). For example, Tris buffers are often used with DEAE exchangers, whilst phosphate and acetate buffers are frequently used with CM exchangers. Moreover, the minimum buffering strength recommended for ion-exchange is approximately 10 m*M* within 0.3 pH units of the buffer dissociation constant or pKa (i.e. the pH at which buffering capacity is strongest). Finally, a buffer pH should be selected that

permits the protein of interest to remain stable, whilst allowing it to bind reversibly to the matrix. It should also be close enough to the pH at which the protein begins to dissociate from the column to prevent the need to adjust the pH or ionic strength during elution to levels that would destabilize the protein.

1.2.3 Selection of adsorption and elution conditions (pH and ionic strength)

Mobile phase pH can be altered to favor either adsorption or elution of proteins. In general, a pH is chosen which will just permit binding of the target protein. This is usually about 1 pH unit above or below the target protein p*I*. A greater difference in pH would lead to stronger protein binding, the need for stronger elution conditions, and decreases in sample resolution and recovery of target protein activity. A pH change can also be used to induce desorption of the target protein (a pH decrease in the case of anion-exchangers and vice-versa). As with pH, mobile phase ionic strength can also be used to control target protein adsorption and elution. As a general rule, the highest ionic strength which will allow adsorption (e.g. 20-50 m*M* NaCl) and the lowest ionic strength which will allow elution are recommended.

1.2.4 Selection of elution format

Two elution formats can be identified: (i) isocratic elution; (ii) gradient elution. With isocratic elution, a single buffer is used throughout the entire separation. Sample components (including the target protein) are only loosely adsorbed to the column matrix. Since individual proteins will have different distribution coefficients, separation is achieved by their relative speeds of migration over the column. To achieve optimum resolution of sample components therefore, a small sample volume (1-5% of the bed volume) and a long exchanger column (1:20 diameter:length ratio) are necessary. Although this technique is time consuming and the desired protein invariably elutes in a large volume, no gradient-forming apparatus is required and column regeneration is usually unnecessary. More commonly, conditions are selected which result in the complete adsorption of the desired protein to the column matrix, necessitating an alteration of eluent conditions to achieve its desorption. With gradient elution, continuous or discontinuous (stepwise) variations in the ionic strength and/or pH of the eluent are used to promote target protein desorption. Whilst stepwise gradients are technically simpler, continuous gradients generally give better resolution.

2. Materials

Unless otherwise indicated, all chemicals can be purchased from Sigma-Aldrich.

2.1 Preparation of Bovine Whole Brain Cytosolic Extract

1. Buffer A: 100 m*M* potassium phosphate pH 7.4, 5 m*M* DTT (dithiothreitol) and 0.5 m*M* EDTA (ethylenediamine tetra acetic acid) [*see* **Note 1**]
2. Bovine whole brain should be obtained from a freshly slaughtered animal. The brain tissue can be sectioned and frozen at -80°C for long term storage.
3. Container with crushed ice
4. Homogenizer (e.g. Sorvall Omni Mixer, standard food blender)
5. Refrigerated centrifuge and rotor (e.g. Beckman J2-MC/ JA-20 rotor; 36,000xg)
6. Refrigerated ultracentrifuge (e.g. Beckman L8-M/ 70Ti rotor; 100,000xg)

2.2 Ammonium Sulphate Precipitation

1. Buffer B: 50 m*M* Tris-HCl pH 8.0, 5 m*M* DTT and 0.5 m*M* EDTA [*see* **Note 2**]
2. Solid $(NH_4)_2SO_4$ (ammonium sulphate)
3. NaOH (sodium hydroxide) at 1 *M*
4. Container with crushed ice
5. Dialysis tubing and sealing clips (Sigma-Aldrich)
6. Glass beakers for both "salting out" and dialysis tube preparation steps
7. Magnetic stirrer and bunsen burner

2.3 Prolyl Oligopeptidase Partial Purification by IEC

1. Buffer B (*see* section 2.2)
2. NaCl (sodium chloride) at 350 m*M* prepared in Buffer B.
3. DEAE-Sepharose® Fast Flow anion-exchange resin (GE Healthcare, Sigma-Aldrich), usually supplied in as pre-swollen beads [*see* **Note 3**]
4. Test tubes for the fraction collector
5. Glass column, low pressure pump (Model EP-1 Econo Pump), fraction collector (Model 2110), silicone tubing and luer-lock fittings (Bio-Rad Laboratories are recommended for all of the aforementioned LC hardware) [*see* **Note 4**]
6. Gradient forming device with 100-200 mL capacity (Sigma-Aldrich) [*see* **Note 5**]

2.4 Assay of Post-DEAE Fractions

1. Buffer A (*see* section 2.1)

2. Bradford and BCA (bicinchoninic acid) protein assay reagents (Bio-Rad Laboratories and Pierce Protein Research Products)
3. BSA protein assay standard at 1 mg/mL (in ultra pure water)
4. Both fluorescent standard (1 m*M* 7-amino-4-methyl-coumarin, MCA) and substrate stock (10 m*M* *Z*-Gly-Pro-MCA) can be prepared in 100% dimethyl sulphoxide (DMSO) and stored as frozen aliquots at -20°C (Bachem)
5. Acetic acid at 1.5 *M* (BDH)
6. Waterbath at 37ºC
7. Fluorescence spectrophotometer (PerkinElmer LS50 is recommended)
8. Glass and quartz microcuvettes, the latter for monitoring absorbance at ≤280 nm

3. Methods

3.1 Preparation of Bovine Whole Brain Cytosolic Extract

All steps to be conducted at 4ºC. Latex gloves should also be worn:

1. Extract preparation has been described previously (5). Briefly, homogenize fully a 50 g whole brain slice in 200 mL of ice-cold Buffer A (*see* section 2.1) [*see* **Note 6**].
2. Centrifuge homogenate for 45 min at 36,000xg to yield a supernatant (S1) and pellet (P1).
3. Resuspend P1 in 100 mL of ice-cold distilled water (osmotic shock step to release occluded POP activity) and recentrifuge as above to yield a second supernatant (S2) and pellet (P2). Discard the P2 pellet.
4. Combine S1 and S2 fractions and ultracentrifuge for 45 min at 100,000xg to yield a whole brain supernatant (S3) for storage as 40 mL aliquots at -20°C/-80°C. Discard the pellet (P3).

3.2 Ammonium Sulphate Precipitation

All steps to be conducted at 4ºC. Latex gloves should also be worn:

1. Add solid ammonium sulphate to 40 mL of S3 with constant stirring to give 45% (w/v) saturation (10.67 g at 4ºC) and adjust to pH 7.4 using 1 *M* NaOH [*see* **Note 7**].
2. Stir for 1 h in a constant gentle manner, and then remove the precipitated ("salted-out") contaminants by refrigerated centrifugation for 45 min at 36,000xg. Following centrifugation, retain the supernatant (S4) and discard the pellet (P4).
3. Add solid ammonium sulphate to S4 with constant stirring to give 75% saturation (9.39 g at 4ºC) and adjust to pH 7.4 using 1 *M* NaOH.

4. Repeat step 2 and retain the pellet (P5). Resuspend P5 in 5 mL of Buffer B to create a "post-ammonium sulphate extract".
5. Dialyse the post-ammonium sulphate extract for 12 h against Buffer B [*see* **Note 8**].

3.3 Prolyl Oligopeptidase Partial Purification by IEC

All steps to be conducted at 4ºC. Latex gloves should also be worn. A column flowrate of 1 mL/min and a fraction collection volume of 5 mL should be used throughout:

1. Equilibrate a 20 mL DEAE-Sepharose® Fast Flow column (diameter: 2.5 cm, height: 3.0 cm) with 100 mL of Buffer B.
2. Apply all of the dialysed post-ammonium sulphate extract (from step 5 above) to the column and wash through the unbound contaminants with 100 mL of Buffer B.
3. Elute bound POP using a 100 mL linear NaCl gradient (0-350 m*M*) prepared in Buffer B [*see* **Note 9**].
4. Regenerate the DEAE column with 60 mL of 350 m*M* NaCl in Buffer B, followed by 100 mL of NaCl-free Buffer B [*see* **Note 10**].
5. Assay (as soon as possible) eluted fractions for total protein and POP activity. Following assay (described below), eluted fractions containing the highest levels of POP activity should be pooled for storage (-20°C) and further purification.

3.4 Assay of Post-DEAE Fractions

Determination of total protein in pre- and post-IEC fractions can be done by monitoring fraction absorbance at 280 nm or using widely available Bio-Rad or BCA standard assay protocols based on the methods of Bradford ***(21)*** and Smith *et al.* ***(22)***, respectively. Determination of POP activity is based on a modification of the original method of Yoshimoto *et al.* using *Z*-Gly-Pro-MCA as substrate ***(23)*** and is described below:

1. Prepare the substrate stock (10 m*M Z*-Gly-Pro-MCA) in 100% DMSO. To 200 μL of substrate stock, add 600 μL of DMSO, followed by Buffer A to a final volume of 10 mL. This will yield a final substrate concentration of 200 μ*M* in 8% DMSO [*see* **Note 11**].
2. Add 400 μL of 200 μ*M* substrate to 100 μL of post-DEAE fraction and incubate at 37°C for 30 min. Fractions should be assayed in triplicate [*see* **Note 12**].
3. Terminate assay reactions after 30 min with 1 mL of 1.5 *M* acetic acid.

4. For blanking purposes, prepare a negative control by addition of 1 mL of 1.5 *M* acetic acid to a 100 μL aliquot of Fraction 1 "prior" to addition of substrate [*see* **Note 13**].
5. Monitor liberated MCA by fluorescence spectrophotometry at excitation and emission wavelengths of 370 and 440 nm, respectively (e.g. Perkin-Elmer LS50). The fluorimeter excitation slit width can be set at 10 nm, whilst the emission slit width can be adjusted (from 2.5-20 nm) to obtain fluorimetric readings within range of a relevant MCA standard curve (0-10 μ*M* or 0-100 μ*M*) [*see* **Note 14**].

4. Notes

1. Potassium phosphate buffer can be prepared from the 100 m*M* "acid" (K_2HPO_4) and "base" (KH_2PO_4) forms of potassium phosphate. The base form can then be adjusted to pH 7.4 using the acid form. Moreover, as DTT loses much of its reducing potency within 12-24 h in solution, it should be prepared (in distilled water) as a concentrated stock (100X) and stored (-20°C), only to be thawed and added to buffers immediately prior to use.
2. Tris-HCl buffer can be prepared from 100 m*M* trizma base and adjusted down to the desired pH with concentrated HCl (hydrochloric acid). Moreover, as Tris buffers are temperature sensitive, they should be adjusted to pH 7.8 when being prepared at room temperature (pH will rise to 8.0 when the buffer is equilibrated to 4°C).
3. Various materials ranging from silica and complex polysaccharides (e.g. dextran, agarose and cellulose) to more complex organic polymers (e.g. polyacrylate, polyvinyl, polyether and polystyrene-divinyl benzene) have been used in the manufacture of ion-exchange resins. Resins are usually porous beads (although fibrous, microgranular and composite matrices are also available), supplied either as dried preparations or in a pre-swollen state to be used in LC applications ranging from bioanalytical monitoring and research to process-scale protein separations. Vendors also provide ion-exchange resins in pre-packed IEC columns and microplates for use with standard LC setups, HPLC systems, and high-throughput applications.
4. Column pouring should be performed at 4°C. Prior to pouring, the column exit valve and tubing should be purged of air with distilled water. A pre-measured volume of the suspended pre-swollen ion-exchange resin can then be poured into the column in "one" pour (this is essential if one is to avoid "gaps" in the resin bed, which can reduce column performance and resolution). The buffer volume or "headspace" above the resin bed

should be kept to a minumum (10-20% of bed volume) in order to ensure accurate delivery of a chosen elution gradient. Moreover, if there is an airspace above the buffer level covering the top of the resin bed (dependent on column dimensions and bed volume), a small piece of parafilm (~1 cm^2) can be placed floating on top of the buffer over the resin. This will act as a "shock absorber" to prevent fluid turbulence (which can cause disruption of the resin bed surface as the buffer is pumped down through the column). Once poured, the column should be washed with several volumes of distilled water to remove preservative.

5. When setting up the gradient maker, ensure that the narrow fluid channel connecting the two gradient compartments is properly purged of air. This can be achieved using a long needle syringe to draw fluid through the channel, or by briefly exerting downward pressure on one of the gradient buffers to force fluid through the channel. Moreover, only gentle magnetic stirring should be used during gradient elution to prevent bubble formation and possible blockage of the gradient fluid channel.
6. Tissue homogenization should be conducted using short, repeated bursts of the homogenizer/blender (i.e. 2-3 sec). This will minimize "shearing" and "foaming", both of which can reduce recovery levels of active enzyme.
7. The "salting out" procedure should be conducted on ice in a small glass beaker placed at the bottom of a small polystyrene dry-ice container (the container can subsequently be placed directly onto a magnetic stirrer).
8. Prior to ion-exchange, samples must be dialysed into the starting buffer (in this case, Buffer B). At least 200 volumes of dialysis buffer are recommended (i.e. relative to the sample volume), with a buffer change after 3-4 h. Dialysis tubing can be prepared by placing a relevant length into a beaker of boiling water containing a large spatula of EDTA (disodium form). This will soften the tubing and remove heavy metal ions. After 5 min in boiling water, the tubing should be rinsed thoroughly in cold distilled water.
9. Linear ionic strength gradients are very reproducible and can be prepared by mixing two buffers of differing ionic strengths in linear volume ratios. Linear pH gradients cannot be prepared by mixing two buffers of different pH values in linear volume ratios due to differences in the buffering capacities of the two buffers being mixed. In addition, the mixed buffer then has to titrate the buffering action of the ion-exchanger and the adsorbed proteins. Consequently, pH gradients are less frequently employed.
10. Avoid leaving high salt buffers on the column for extended periods following regeneration as this may lead to salt crystallization (which necessitates column

repouring). Moreover, for medium-to-long-term storage, the column should be regenerated in the normal fashion, washed in several volumes of distilled water, followed by several volumes of either 0.02% (v/v) sodium azide or 20% (v/v) ethanol.

11. When diluting the 10 m*M Z*-Gly-Pro-MCA stock, Buffer A should be pre-warmed and added very slowly to the final volume of 10 mL. Moreover, the DMSO should be added in 100 μL increments in parallel with the buffer to prevent the substrate precipitating out of solution. The 200 μ*M* substrate should be prepared from stock only as required.
12. Prior to assay, both fraction triplicates and substrate should be pre-equilibrated to 37°C. At t=0, the substrate should be added to sequentially numbered fractions at exactly 15 sec intervals. At t=30, the acetic acid should be added to fractions at exactly 15 sec intervals. In this way, all fractions receive the exact 30 min assay time.
13. When assaying any sample for POP activity, the appropriate negative control should always be; Sample (crude or purified), followed sequentially (at t=30 min) by Stopping Agent (1.5 *M* acetic acid) + Substrate (200 μ*M Z*-Gly-Pro-MCA).
14. The MCA standard curve must be prepared under identical assay conditions and read at the same fluorimeter settings as those used for the assay in order to properly quantitate MCA release for use in the calculation of enzyme activity expression. Units of POP activity are defined as picomoles of MCA released per min at 37°C.

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Figure Captions

Fig. 1. Ion-exchange chromatography schematic. It is the nature of the counterions displaced from the matrix functional groups (M^+, M^-) which determines the IEC format. Thus, with anion-exchange chromatography, the stationary phase (usually a porous bead) displays a positively charged functional group with counter anion (A^-) that can be displaced by an anionic solute (S^-). By contrast, with cation-exchange chromatography, the stationary phase displays a negatively charged functional group with counter cation (C^+) that can be displaced by a cationic solute (S^+).

Fig. 2**.** Partial purification of prolyl oligopeptidase (POP) from bovine whole brain cytosol via DEAE-Sepharose® Fast Flow anion-exchange chromatography. Dialysed post-ammonium sulphate POP fraction (7.4 mL) was applied to a pre-equilibrated 20 mL DEAE-Sepharose® column (50 m*M* Tris-HCl pH 8.0, 5 m*M* DTT, 0.5 m*M* EDTA). Following removal of unbound contaminants, bound POP was eluted with a linear NaCl gradient (- - - -) from 0-350 m*M*. 5 mL fractions were collected and assayed for POP activity (-●-) via fluorimetric assay at excitation and emission wavelengths of 370 and 440 nm, respectively (fluroescence intensity values plotted). Protein (·····) was monitored by BCA microplate assay at 595 nm. Peak POP elution occurred at 180 m*M* NaCl. All data courtesy of Dr. Brendan O'Connor (unpublished observations). DTT: Dithiothreitol, EDTA: Ethylene diamine tetraacetic acid, NaCl: Sodium chloride.

Table Captions

Table 1. Overview of commercial ion-exchanger properties.

Table 2. Partial purification of prolyl oligopeptidase (POP) from bovine whole brain cytosolic fraction. Enzyme activity (units) expressed as picomoles of MCA released (i.e. from *Z*-Gly-Pro-MCA) per min at 37ºC. Protein (mg) was determined by BCA microplate assay. All data courtesy of Dr. Brendan O'Connor (unpublished observations).