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PROTEIN PURIFICATION AND ITS APPLICATION TO CRYSTALLIZATION

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CHAPTER 1

INTRODUCTION

Structural studies on proteins depend on an investigator's ability to isolate and purify the protein. In many cases, protein isolation is a trivial matter, but purification to homogeneity is a lengthy process. Traditionally, proteins have been purified by a combination of precipitation, open-column chromatography, including size-exclusion and ion-exchange chromatography, ultracentrifugation, and electrophoresis. Recently high-performance liquid chromatography (HPLC) has become increasingly important in the purification and analysis of proteins (1-3). The purpose of this study was to apply modern HPLC techniques to the protein purification problems encountered in crystallographic studies.

1.1 PROTEIN CRYSTALLIZATION

Successful x-ray structure analysis is dependent on the growth of suitable 'single crystals' of the protein (4). Successful crystallization of a particular protein is directly related to the purity of that protein. 'Hyper-purified' proteins are thought to yield crystals of a higher quality than less highly-purified preparations of the same protein (5). 'Hyper-purification' refers to any purification technique that offers high resolution, but is generally limited to isoelectric-focusing and HPLC. HPLC has several advantages as a 'hyper-purification' technique: 1) several modes of separation are available (e.g. ion-exchange, hydrophobic interaction, and size-exclusion); 2) the separation is rapid, which can be important when handling unstable or easily degraded proteins; 3) the selectivity of a particular separation can be altered by adding various organic modifiers to the separation solvents.

The Laboratory for the Structure of Matter is involved in a broadly-based program aimed at the crystallization, x-ray structure analysis, and modeling of a variety of proteins. The investigators have prepared single crystals of a variety of proteins, and have successfully used the batch method, bulk dialysis, micro-dialysis, and micro-vapor diffusion techniques. During the period of this study, HPLC techniques were integrated into the standard laboratory practices to ensure that samples being used in crystallization

experiments were of sufficiently high purity, and to 'hyper-purify' samples which were not already homogeneous. An attempt was also made to integrate HPLC purification and protein crystallization experiments through a novel 'chromatographic' crystallization method.

ACKNOWLEDGEMENTS

1.2 ABREVIATIONS USED IN THIS REPORT

ACE	angiotensin converting enzyme
AChE	acetylcholinesterase
DFP	diisopropylfluorophosphate
DFPase	diisopropylfluorophosphate fluoro-hydrolase (old name for OPAase)
FPLC	fast-protein liquid chromatography (a product name from Pharmacia Inc.)
HIC	hydrophobic interaction chromatography
HLFA	human lymphocyte function-antigen
HPLC	high-performance liquid chromatography
IEC	ion-exchange chromatography
k Da	kiloDaltons
OPAase	organo-phosphorous acid anhydrase
RPC	reversed-phase chromatography
SEB	Staphylococcal enterotoxin B

1.3 REFERENCES

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CHAPTER 2

PROTEIN PURIFICATION

2.1 DETERGENT EFFECTS

Chromatographic systems for the high-performance liquid chromatography (HPLC) of hydrophobic, or membrane, proteins are generally limited to size-exclusion or ion-exchange chromatography. Attempts at reversed-phase chromatography of these proteins frequently results in either low recovery, or no recovery, of the protein.

2.1.1 Background

Denaturing agents, such as 8M urea of 6M guanidine HCl, or strong detergents are required for the solubilization of membrane proteins and other hydrophobic proteins. It has been noted that "... these solvent systems are useful in size-exclusion chromatography (SEC), but not in reversed-phase chromatography" (1). Detergent can cause problems in size-exclusion chromatography through the formation of micelles, but does not always interfere with the separation. Detergent is far more deleterious in ion-exchange chromatography where ionic detergents can poison the column. Although the literature contains some examples of the reversed-phase HPLC of membrane proteins "...a general method [such as reversed-phase HPLC for soluble proteins] that can be applied to the purification of labile, membrane-bound enzymes, still remains an elusive goal" (2).

Interactions between the proteins, solvent, and stationary-phase lead to various types of 'non-ideal' behavior in the reversed-phase HPLC of proteins. It is these 'non-ideal' interactions that make a general separation method an "elusive goal". Non-ideal behavior can range from denaturation (3-6) and ghost peaks in subsequent elutions of the column (7,8) to loss of protein (7,9). The generally accepted explanation for this non-ideal behavior is complex multi-site interactions between the protein and column packing. Further evidence on the 'multi-site' nature of interactions between proteins and reversed-phase columns can be found in the relationship between chromatographic resolution of a mixture of proteins and column length. In the ideal case, an increase in column length results in a concomitant increase in

resolution; this is not the case with proteins. Reductions in column length over the range 25 cm to 1 or 2 cm result in virtually no change in resolution (10). The stainless-steel frit, at the column inlet, has also been implicated in protein loss (9), although active sites on the column can also play a role in the loss of protein.

2.1.2 Experimental Design

In an attempt to overcome the poor mass recovery problems associated with the reversed-phase HPLC of hydrophobic proteins, the effect of detergent on the separation of a variety of hydrophilic and hydrophobic proteins was studied. mini-columns, 10 mm in length, were packed with a variety of reversed-phase matricies. The columns were tested with a mixture of water soluble proteins to evaluate chromatographic resolution, sample recovery, and peak shape. Following this test with 'standard' proteins, the column was saturated with laboratory grade detergent, such as Brij 35, Brij 99 or octylglucoside, and re-tested with the 'standard' proteins in the presence of detergent. Finally, the columns were challenged with either red blood cell acetylcholinesterase (AChE) or human lymphocyte function-antigen (HLFA).

2.1.3 Results And Discussion

Three different matricies were tested in this study, one of these was a small-pore silica, and the other two could be described as large-pore silicas. The retention times of proteins on the large-pore C-8 matrix were shorter than those on the large-pore C-18 matrix, as would be predicted based on the difference in hydrophobicity of the two matricies. In comparong the two C-18 matricies, resolution appeared to be slightly improved on the large-pore silica matrix as compared to the small-pore matrix. After exposure to detergent, a large shift in retention time was observed, but only for the large-pore matricies, and only for one of the test proteins. No similar shift was observed on the small-pore matrix. A slight increase in efficiency was observed in the presence of detergent. This increased efficency could not be attributed only to the decreased denaturation of the protein, which should result in shorter retention times, ss this was observed for only one protein in the test mixture. Additionally, the improved efficiency can not be attributed to reduced peak width, as peak width was not significantly affected by detergent. Peak shape, or more specificly peak asymmetry, was improved by the addition of detergent. This is likely due to a decrease in the magnitude of the multi-site interactions, as these interactions can cause peak tailing resulting in highly asymmetric peaks and reduced resolution.

Column capacity varied with the nature of the matrix, but was in the range of 100 to 150 micrograms. Column capacity did not seem to be affected by the addition of detergent. Reproducibility

of both retention time and the ratio of peak area to protein load was excellent.

Tests involving integral membrane proteins, i.e. HLFA or AChE, were also encouraging. Prior attempts at reversed-phase chromatography of AChE resulted in no recovery of protein. Based on enzyme activity, 40 to 60% of the AChE activity could be recovered from a mini-column saturated with octylglucoside. Additionally, some resolution of this affinity-purified protein into its components was achieved. No attempt was made to change the solvents or gradient program to improve recovery of activity. HLFA chromatographed as a single peak, and was used to measure recovery of an integral membrane protein. In the range tested, recovery of HLFA varied from 87 to 103% (average 96.4%), which was comparable to the recovery achieved with non-membrane proteins under similar conditions (i.e. 95.2%)

2.1.4 Detergent Effects Summary

Integral membrane proteins were successfuly chromatographed on reversed-phase mini-columns in the presence of detergent. HLFA exhibited recovery similar to that of non-membrane proteins. Although as much as 66% of the red blood cell AChE activity was recovered, ghost peaks were observed in subsequent elutions of the column (11). The enhanced chromatographic efficiency observed in this study could not be wholly attributed to decreased peak width. This result is in agreement with prior studies on the chromatography of proteins on mini-columns in the absence of detergents (10). The efficency enhancement has been attributed to a combination of decreased denaturation and decreased multi-site interactions unrelated to denaturation as decreased denaturation alone would generally result in decreased retention times.

2.2 SQUID ORGANOPHOSPHOROUS ACID ANHYDRASE

2.2.1 Introduction

Cephalopod optic ganglion and hepatopancrease contain an organic phosphorous acid anhydrase (OPAase) which hydrolyzes diiso-propylfluorophosphate (DFP) and soman, releasing fluoride and detoxifying these nerve agents. Earlier reports have referred to this enzyme as a diisopropylfluorophosphate fluorohydrolase (DFPase) (12,13), which is not wholly accurate in describing the enzymatic activity, nor does it convey the relationship between this enzyme and other OPA enzymes (12-16).

Because of the ability of this enzyme, and related OPAases to detoxify various nerve agents, such as DFP, soman, and insecticides, much of the literature describing these enzymes is devoted to the kinetics of detoxification, and comparative studies on different enzymes and their effectivness in degrading various organophosphorous substrates (13,16, 18-20). In this report, an

improved purification for the enzyme and data on the biochemical characterization of the enzyme are presented.

2.2.2 Purification Of Squid OPA

A crude preparation of squid organophosphorous acid anhydrase (OPA) was isolated from hepatopancrease by precipitation with ammonium sulfate followed by size exclusion chromatography, using a sephacryl S-200 column, as previously described (12,13). The pooled OPA containing fractions were then concentrated using an Amicon YM-10 ultrafiltration membrane, and the concentrated enzyme washed with 50 mM phosphate buffer (pH 7.0). The enzyme was further purified by anion-exchange high-performance liquid chromatography (HPLC) on a Toya Soda DEAE-5PW column (17) and hydrophobic interaction chromatography (HIC) on a Beckman CAA-HIC column (Figures 2.1 and 2.2). The HPLC system consisted of a Beckman model 336 binary gradient HPLC equipped with a Kratos Spectraflow-783 variable wavelength detector. A summary of the purification appears in Table 2.1.

TABLE 2.1

Summary of Purification of Squid OPAase (data compiled from three different purifications).

Purification Step	Protein mg	Activity total U	U/mg	per cent Yield	Fold Purification
Tissue Homogenate	17000	1507	0.089	---	-----
45% Amm Sulf supernantant	14400	1724	0.120	114	1.35
75% Amm Sulf precipitate	630	553	0.880	37	9.89
SEC Pool	354	517	1.460	34	16.40
IEC Pool	105	425	4.047	28	45.47

2.2.3 Characterization Of Squid OPA

Purified squid OPA was subjected to isoelectric-focusing using an LKB Multiphore II apparatus and precast 'Amopholine Pagplates' (LKB Bromma) with a pH range of 3.5 to 9.5. After electrophoresis,

FIGURE 2.1. HPLC anion-exchange chromatography of squid
          hepatopancrease OPAase and activity profile of the
          collected fractions expressed as the change in
          response of the fluoride electrode in mV. Note
          that the peak in activity corresponds to a portion
          of the chromatogram which contains relatively
          little protein.

Change in mV (rel mV)

-60

-40

-20

0

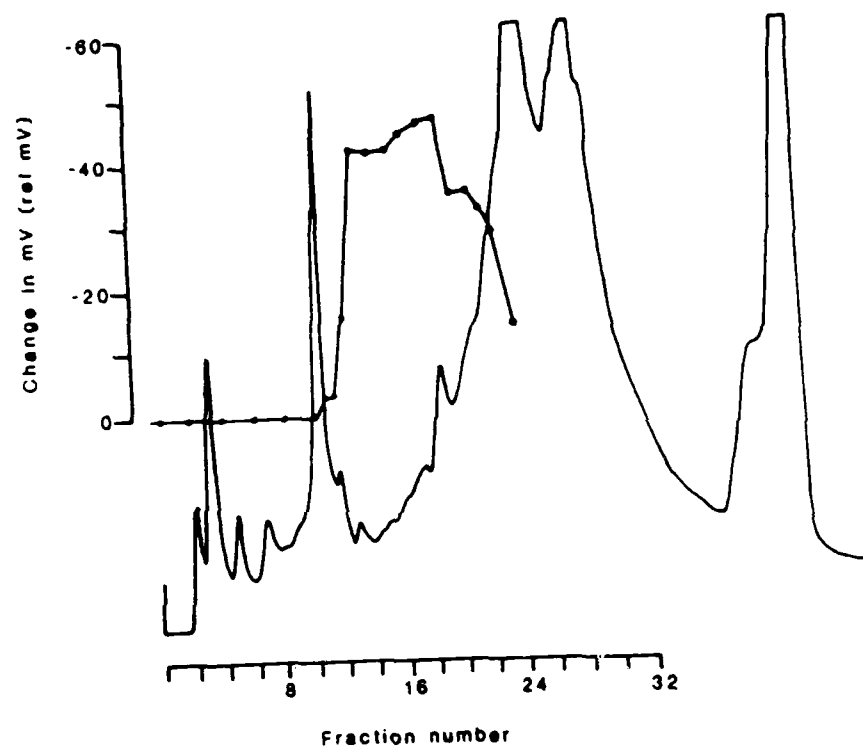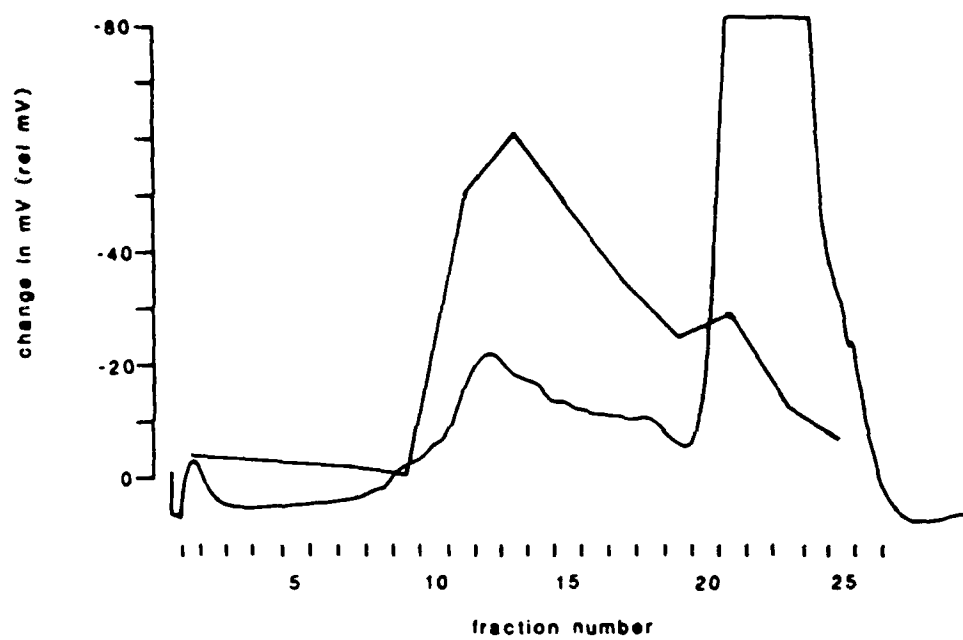8   16   24   32

Fraction number

FIGURE 2.2. HPLC hydrophobic-interaction chromatography
of partially purified squid hepatopancrease OPAase
and activity profile of the collected fractions.

the protein bands were visualized by silver staining and the isoelectric points estimated by comparison to standard proteins on the same gel. The results of these experiments showed two distinct forms of the OPA enzyme are present in hepatopancrease tissue with isolelectric points of about 5.07 and 5.13. This is similar to the isoelectric point reported for OPA from optic ganglion, 5.2, but does not compare well with the previously reported isoelectric point of 5.5 for OPA from squid hepatopancrease (13). It should be noted that we now know this enzyme to be dependent on the presence of metal ions, and removal of these ions causes a shift in the isoelectric point to a higher pH. It is not known if the two forms of hepatopancrease OPA observed represent multiple molecular forms of OPA or were an artifact of the purification method.

HPLC size-exclusion chromatography, using a TSK 3000-SW column (Toya Soda Inc.), was used to mesure an apparent molecular weight of the OPA. The results of these experiments indicated an apparent molecular weight of about 26,000 daltons, which compares well with earlier results (15).

2.2.3.1 Effect Of pH On OPAase Activity

The effect of pH on enzyme stability is important during the purification of the enzyme. Additionally, a 'true' measure of enzyme activity can only be made if the relationship between pH and activity is known, as an activity measurement made at the wrong pH could yield either no activity or low activity leading to false conclusions regarding specific activity and total enzyme present. Therefore, the effect of pH on enzyme stability and enzyme activity was measured by preincubating an aliquot of enzyme at a given pH and assaying the preincubated enzyme either at a standard pH to determine enzyme stability or at the incubation pH to determine the relationship between pH and activity.

2.2.3.2 Compositional Analysis

Amino acid compositional anaysis was performed on a Beckman model 6300 amino acid analyzer after acid hydrolysis. The results of four independent determinations were averaged and the results presented in Table 2.2.

2.2.3.3 Determination Of Extinction Coefficient

The molar extinction coefficient, and E1%, were determined from the UV absorption. For squid hepatopancrease DFPase, E1% was determined to be 15.5, and the molar extinction coefficient, based on a molecular weight of 26,000, was determined to be 38,500. The molar extinction coefficient of a protein can be estimated from the

TABLE 2.2

**Amino Acid Compositional Analysis of Squid
Hepatopancrease OPAase.**

amino acid	number residues	2 x number residues	round off
cys acid	5.4	10.8	11
asx	13.0	26.0	26
thr	8.1	16.2	16
ser	3.7	7.4	7
glx	10.9	21.8	22
pro	3.8	7.6	8
gly	11.6	23.2	23
ala	6.7	13.4	13
cys/2	0.0	0.0	0
val	7.3	14.6	15
met	0.1	0.2	0
ile	4.5	9.0	9
leu	6.3	12.6	13
tyr	0.9	1.8	2
phe	5.2	10.4	10
his	2.4	4.8	5
lys	5.5	11.0	11
arg	4.1	8.2	8

number of tryptophane (Trp) and tyrosine (Tyr) residues using the equation:

molar extinction coefficient = (5960 x No. Trp) + (1280 x No. Tyr)

If the extinction coefficient is known, then the number of tryptophan residues in a protein can be estimated using this same equation. Based on the experimentally determined molar extinction coefficient of squid DFPase, and the results of the compositional analysis, it was estimated that there are 6 tryptophan residues per molecule of squid hepatopancrease OPA.

2.2.4 OPAase Summary

One interesting observation is that there are apparently two forms of DFPase which can be recovered from hepatopancrease tissue. Until further work is done, it is not known whether these enzymes constitute two unique enzymes, or precursor-final product of one enzyme; it is also possible that one form is an artifact of the purification. Both forms show activity and were separated by using different starting conditions on the HIC HPLC step. These enzyme forms differ slightly in their pI.

2.3 ELAPID ACETYLCHOLINESTERASE

2.3.1 Introduction

Acetylcholinesterase (AChE) is primarily associated with neu-ronal cells, but has been isolated from a number of other sources including erythrocytes, and various organs (21,22). AChE hydrolyzes the ester linkage in acetylcholine releasing acetate and choline at the postsynaptic membrane, thus restoring the polarized state, and excitability, of the postsynaptic membrane (23). The proper function of AChE is necessary for normal neuronal transmission.

On the basis of enzymatic activity, substrate specificity, and the effect of various inhibitors, the enzymes isolated from various sources have been classified either as 'true cholinesterases', or 'pseudocholinesterases'. 'True cholinesterases' hydrolyze acetylcholine more rapidly than other choline derivatives. 'Pseudocholinesterases', sometimes called plasma cholinesterase or butyrylcholinesterase, are less specific and hydrolyze succinylcholine more rapidly than the true cholinesterases do. Only the true cholinesterases exhibit substrate inhibition.

Soluble forms of AChE have been found in the venoms of several elapids (i.e. cobras and krates), and their specificity studied (24-29). A comparison of eel electroplax AChE, a membrane-bound 'true cholinesterase', with the soluble cholinesterase found in elapid venom revealed many similarities, including substrate inhibition, between the enzymes (24). The soluble cholinesterase found in elapid venom is therefore a convenient model of the membrane bound cholinesterases.

Acetylcholinesterase is present in concentrations as high as 1.7 mg/g dry venom of *Naja naja oxiana*. As many as 15 different isoenzymes of AChE have been isolated from one elapid venom (28). The large number of isoenzynmes may be the result of proteolytic activity as proteases are also in the venom; it is therefore essential that the purification be carried out quickly so as to minimize contact with the proteases.

Previously AChE has been isolated from elapid venom by a combination of chromatographic techniques. The final purification step is generally affinity chromatography. Recently, a high performance affinity procedure for the isolation of acetylcholinesterase has been described (B.P. Doctor, unpublished; Sam Morris, personal communication). This new affinity procedure was modified for batch chromatography and tested for use in isolating elapid venom AChE.

2.3.2 EXPERIMENTAL METHODS

2.3.2.1 Preparation Of Affinity Support

A Beckman Ultraffinity-EP column capacity kit was activated with Procainamide-HCl, U.S.P. grade (Unit Dose Laboratories, Inc.) as follows: 2 ml of a solution containing 5 mg/ml procainamide-HCl in 1 M potassium phosphate, pH 6.8 was injected into the vial containing the affinty support (0.1g). The vial was agitated until all of the affinity support was suspended. The suspension was set aside at room temperature for 24 hours. The derivatized affinity support was washed 3 times with 1 ml distilled water followed by 1 ml 95% ethanol. The derivatized affinity support was stored in 95 % ethanol.

2.3.2.2 Binding Tests

Initial tests of binding were performed by pipeting a known quantity of AChE activity, from *Naja naja kaouthia* venom fraction, into a tube containing the affinity support, suspending the affinity support in the AChE containing solution, centrifuging, and measuring the amount of activity left in solution. The support was prepared for the next experiment by washing with 1 ml of 100 mM decamethonium bromide (Aldrich Chemical Co.), to remove bound enzyme, followed by washing with 3 1 ml aliquots of 0.1 M phosphate buffer (pH 7.0) to re-equilibrate the affinity support.

2.3.2.3 Enzyme Purification

Naja naja kaouthia AChE was purified by placing 0.5 ml of a venom fraction from that species into a tube containing the affinity support. After vigorous mixing the mixture was allowed to stand for one minute, centrifuged, and the affinty support washed with 1 ml of 0.1 M phosphate buffer (pH 7.0). The product was then eluted from the support with 1 ml of 100 mM decamethonium bromide, and after centrifugation, the affinity purified AChE decanted. This procedure was repeated five times and the affinity-purified AChE pooled. The purified enzyme was then concentrated in a centricon (Amicon Inc.), and washed with 10 ml of 50 mM phosphate buffer (pH 7.0), in 2 ml aliquots, to remove the decamethonium bromide. *Naja naja atra* AChe was purified from 1 ml of a solution containing 3.1 mg dry venom (Sigma Chemical) as described above. The washing of non-binding or weakly associated protein was extended to four 1 ml aliquots of phosphate buffer.

2.3.3 Characterization Of Affinty Purified AChE

Cholinesterase activity was determined using a colorimetric method (30) with a Shimadzu UV-260 spectrophotometer. The purified AChE was treated with 25 and 250 micromolar decamethonium bromide

and the inhibition of enzymatic activity determined. Total protein was determined by the Bradford dye binding assay (31), and specific activity calculated from these data.

The affinity purified AChE was further characterized by high-performance size-exclusion chromatography on a TSK 2000-SW column (Toya Soda Inc.) and on a Superose-12 column (Pharmacia Inc.), and by reversed-phase HPLC on an Ultrasphere (Beckman Inst. Inc.). The HPLC system consisted of a Beckman model 336 binary gradient HPLC equipped with a Kratos Spectraflow-783 variable wavelength detector.

2.3.4 RESULTS AND DISCUSSION

2.3.4.1 Binding Study

The results of the binding stidies are sumarized in Table 2.3. From the binding assays, the capacity of the affinity support in the test kit was estimated to be about 1000 units and thus about 10,000 units (i.e. 2 to 3 mg) could be bound on a small Ultraffinity-EP column. As the amount of enzyme applied to the affinity support approaches the maximum binding capacity, the amount of enzyme retained approaches 50 to 60 per cent of the activity applied.

TABLE 2.3.

Summarized results on the binding of cobra venom acetylcholinesterase to the procainamide derivatized 'Ultra-affinity' support.

UNITS Applied	UNITS Bound	per cent Bound
1.77	1.71	96
17.7	15.9	90
17.7	16.2	92
17.7	16.0	90
24.4	14.3	59
186	99.0	53
373	101	27

2.3.4.2 Affinity Chromatography

The batch purification of 3 ml of a cobra venom fraction, containing about 1110 units AChE, resulted in a 53 per cent yield of affinity purfied AChE (see Table 2.4). The product had a specific activity of 193 units/mg which represents a 12-fold purification.

Batch purification of AChE from 1 ml (3.1 mg) of *Naja naja atra* venom produced an enzyme with a specific activity of 2667 U/mg (Table 2.5) which represents a 338-fold purification in one step. The yield of 47 per cent (based on activity recovered) is comparable to the yield of AChE from *Naja naja kaouthia*, and to the yield observed in prior studies (Sam Morris, unpublished). Based on the yield of cholinesterase activity and the specific activity of the isolated AChE, it is estimated that *Naja naja atra* venom contains approximately 0.8 mg AChE per gram dried venom.

TABLE 2.4.
Batch affinity purification of acetylcholinesterase
from *Naja naja kaouthia* venom fraction

SAMPLE	FRACTION VOLUME(ul)	ACTIVITY U/ul(a)	TOTAL Units	PROTEIN (ug/ul)	SPECIFIC ACTIVITY
Crude AChE	3000(b)	0.37	1110(c)	23.9	15.4
affinity AChE	1000	0.60	600	3.1	193

(a) activity = slope/3.67. see: Rosenberry and Sloggin, 1984.

(b) total amount of sample applied to affinity support in 6 separate batch extractions (i.e. 6 x 0.5 ml).

(c) total activity = (units x (1/dilution factor) x fraction volume)/ sample size (i.e. 10 ul).

TABLE 2.5.
Batch affinity purification of acetylcholinesterase
from *Naja naja atra* venom.

SAMPLE	FRACTION VOLUME(ul)	ACTIVITY U/ul(a)	TOTAL Units	PROTEIN (ug/ul)	SPECIFIC ACTIVITY
venom	1000	0.024	24.4	3.100	7.9
affinity AChE	250	0.050	11.6	0.017	2667

Characterization of the affinity purified *Naja naja kaouthia*
AChE, by both size-exclusion and reversed phase HPLC, revealed that
the sample was nonhomogeneous. Further investigation showed that a
minimum of three washes of the affinity matrix prior to elution of
the protein was needed to remove all of the non-bound and weakly
bound protein (Figure 2.3). As a result of this finding,
additional wash steps were added to the purification of *Naja naja
atra* AChE.

The affinity purified AChE from *Naja naja atra* showed some
high molecular weight contaminants, i.e. 120-140 k Da (Figure
2.4), which appear to be enriched in the affinity fraction. These
high molecular weight components may be aggregation products of the
AChE, as AChE from other sources is known to aggregate in the
absence of detergent (32). It should also be noted that the
molecular weight of elapid venom AChE, i.e. 67 k Da, is the
approximate molecular weight of the monomer from human erythrocyte
AChE after removal of the hydrophobic domain (32). The high
recovery of activity and speed of this method make it an excellent
choice for the isolation and purfication of AChE.

2.3.5 HPLC Screening Technique

The results of preparative chromatography are often evaluated
by gel electrophoresis, most commonly SDS-PAGE, although other
electrophoretic methods are sometimes used (e.g. native gels or
isoelectric-focusing). SDS-PAGE is useful in characterizing
fractions based on their molecular weight, but requires 2 to 4
hours to prepare the gel, 1 to 3 hours to run the gel, and
additional time to stain the gel. The results of this method are
qualitative, and generally the analysis is performed after all of
the fractions are available due to the difficulty sometimes
encountered in comparing gels. By using high performance liquid
chomatographic (HPLC) methods, fractions from preparative or batch
chromatography can be characterized as they are collected, the
analysis time is shorter (see Figure 2.5), results are easily
quantified by comparing peak area or height, and there is very high
run-to-run reproducibility. Additionally, HPLC methods are easily
automated.

To evaluate the effectivness of the affinity purification
samples of crude venom, the non-binding fraction, the various
washes performed on the bound cholinesterase, and the affinity
purified cholinesterase were chromatographed on an HPLC
size-exclusion column. Using this technique, the purity of the
product was evaluated and loss of cholinesterase, due to leaching
from he affinity matrix, was traced throughout the batch
purification proceedure. This HPLC sceening procedure was also
successfully applied to the preparative chromatography of
cardiotoxin.

FIGURE 2.3.   HPLC size-exclusion chromatography of succes-
            sive washes of the affinity support after applica-
            tion of approximately 350 units *Naja naja kaouthia*
            acetylcholinesterase, and the eluted product after
            two washes.

wash 1

wash 2

elution

0.002 AU

Elution Volume (ml)

2-17
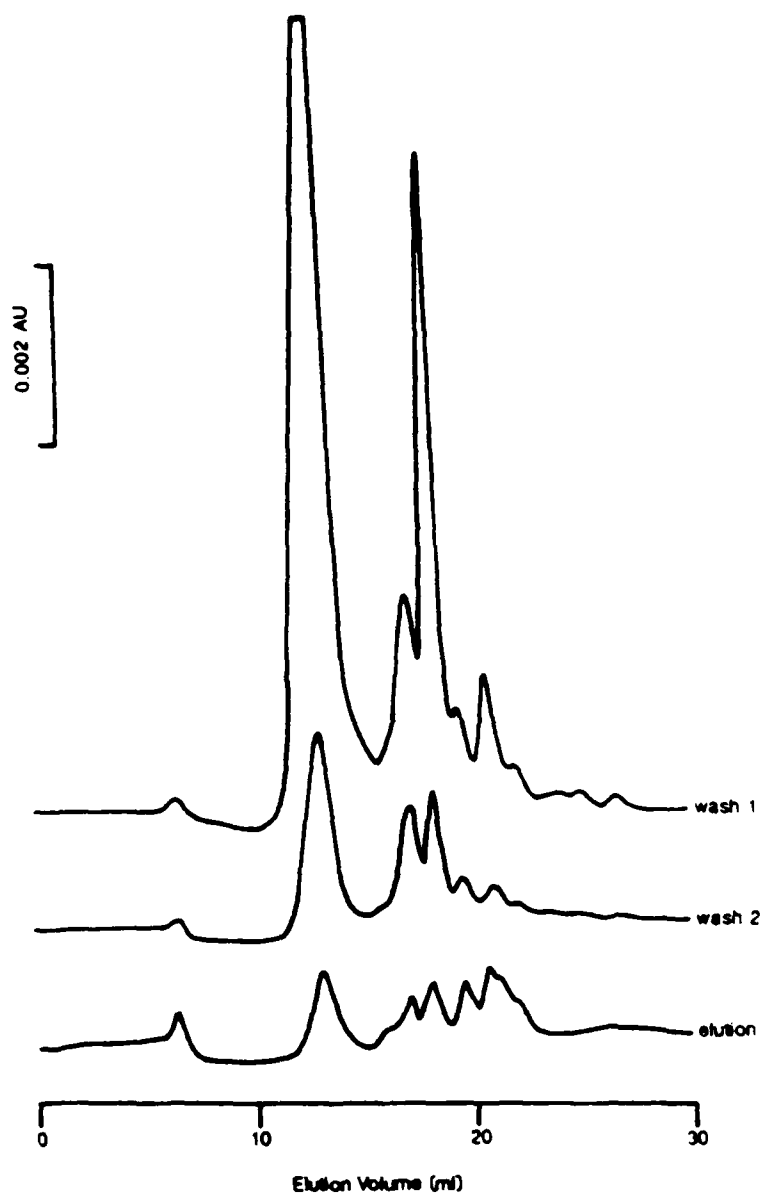
FIGURE 2.4.   HPLC size-exclusion chromatography tracing the
              purification of acetylcholinesterase from *Naja naja
              atra* venom from the venom, through the non-binding
              fraction and wash, to the affinity purified enzyme.

0.002 AU

venom

non-binding

wash 2

affinity AChE

Elution Volume (ml)
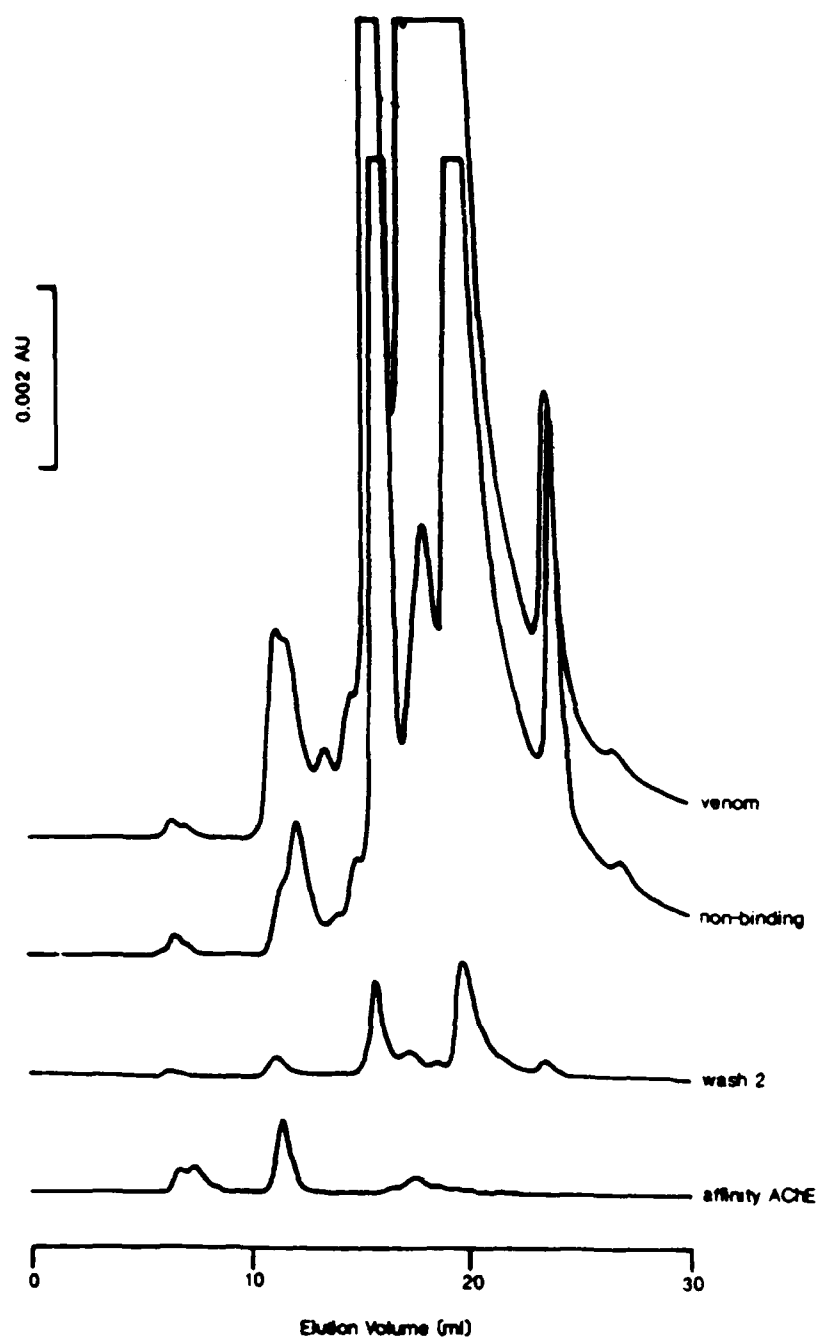
FIGURE 2.5. A comparison of the analysis time required
        for SDS mini-gels vs. analytic HPLC for screening
        fractions from affinity or preparative chromato-
        graphy. Note: operations in italics are 'optional'
        depending on the method used (e.g. pre-cast gels
        can be used, and thus eliminate the time required
        for gel casting and polymerization; the use of
        isocratic elution eliminates the need to re-
        equilibrate the column in HPLC analysis).

	SDS-PAGE		HPLC	
cumulative time (hours)	operation		operation	cumulative time (hours)

SDS-PAGE

cumulative time (hours)	operation
◑	*pour gel*
● ◔	*gel polymerization*
● ◐	sample preparation
● ◕	load samples
● ● ◕	run gel
● ● ● ◔	fix gel
● ● ● ◕	stain gel
variable	*destain gel*

HPLC

operation	cumulative time (hours)
sample preparation	◷
load sample	◷
elute column	◐
equilibrate column	◕

2.3.6 Elapid Cholinesterase Summary

Acetylcholinesterase (EC 3.1.1.7) was isolated by batch affinity chromatography *from* the venom of the elapids *Naja naja kaouthia* and *Naja naja atra* using procainamide-HCl linked to a high-performance affinity matrix. The binding capacity of the derivatized affinity support was 1,000 units cholinesterase/ gram support. Affinity purified acetylcholinesterase (AChE) was analyzed for purity and specific activity.

A single step affinity purification of cobra venom AChE yields about a 340 fold increase in purity. The purified enzyme has a specific activity of over 2600 U/mg, and about 50% of the activity applied to the column is recovered in the product. The utility of size-exclusion HPLC as a tool for screening batch affinty fractions was demonstracted. Size-exclusion HPLC of the affinity purified AChE reveals a nearly homogeneous product with a molecular weight of 67 k Da and small quantities of higher molecular weight contaminants.

2.4 REFERENCES

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CHAPTER 3

APPLICATION OF HPLC TECHNIQUES TO CRYSTALLIZATION

3.1 COLUMN CHROMATOGRAPHIC METHOD

.p Our laboratory has developed a novel method for automating crystallization trials which employs laboratory robotics and automated image analysis (1-4). A new method is currently being developed which will make use of technology developed in the robotics and image analysis project and extend the range of automated procedures to include the conditions required for the crystallization of membrane proteins. A proposal has been written to NIH requesting additional funds to allow us to extend our current efforts into the field of membrane transport proteins. We intend 1) to devolop methods of general applicability to crystallize such proteins; 2) to characterize the crystals obtained as a result of these efforts.

3.1.1 Introduction

All stategies for crystallization of macromolecules are aimed at bringing a concentrated solution of a homogeneous population of protein molecules very slowly toward a state of minimum solubility. The goal is to achieve a limited degree of super-saturation, from which the system can relax by formation of a crystalline precipitate. Generally, the solubility minimum is achieved by modifying the properties of the solvent either through equilibration with precipitating agent or altering some physical variable such as temperature. Many techniques developed for achieving these ends have been described (5,22).

A new method of crystallization of proteins uses chromatography to prepare saturated solutions of proteins which are then allowed to crystallize (6). This method purports to accelerate protein crystallization and avoid conditions of over-saturation which lead to the formation of amorphous precipitate, but as described it is not useful for micro-crystsallization experiments as over 1 mg of protein is required per experiment.

Future crystallographic studies will focus on integral membrane proteins, with emphasis on transport proteins and receptors. However, membrane proteins often chromatograph poorly, and only a few membrane proteins have been crystallized. Recent advances combining 'mini-columns' (7) and detergents have lead to the development of HPLC methods for the purification of membrane proteins (8). Therefore, a chromatographic procedure employing HPLC 'mini-columns' with the addition of detergents to the solvents, as required by protein solubility, is being developed for the preparation of saturated solutions of these proteins.

Saturated solutions of protein, prepared chromatographically, can be stored at room temperature until crystals form, as described by Gulewicz et al. (6). In those cases where this technique fails to produce crystals, the saturated protein solution can either be transferred to a 'hanging-drop vapor diffusion' experiment, in which the concentration of one (or more) of the solvent system components is slowly changed to reduce the solubility of the protein, or the temperature can be altered to try and reduce protein solubility. A variety of solvent systems will be investigated. This HPLC technique will eliminate the need for large quantities of protein (i.e. 1 mg or greater) for each experiment. An additional benefit of the HPLC system is the use of microprocessor control which will allow the automation of a series of experiments which would otherwise be time consuming.

The use of an HPLC system for the preparation of saturated solutions of protein has the advantage of using relatively small amounts of protein (i.e. about 100 ug with conventional column technology). Although this is more than the 20 to 40 ug of protein used in micro-vapor diffusion experiments, there are several advantages to using the chromatographic system: 1) as a gradient is used in eluting the proteins from the column, phase diagrams can be rapidly constructed to predict protein solubility and conditions that may be favorable to crystallization; 2) additional purification of the protein will occur even on the 'mini-columns' described in this proposal producing a more homogeneous population of molecules which is desirable for crystallization; 3) the entire process can be automated thus freeing the scientist to concentrate on experimental design, and not the physical process of setting up crystallization experiments. Finally, it should be noted that modern column technologies can be used to further decrease the amount of protein required for each experiment and thus narrow the gap between the amount of protein required by micro-vapor diffusion experiments and 'chromatographic crystallization' experiments.

3.1.2 NIH Proposal

In the proposal prepared for NIH, crystallization efforts will be focused on the anion transport protein of human red blood cells, identified as Band 3 (9-12). This ion-transport protein has been implicated in cystic fibrosis (13). The lac transport protein of *E. coli* (14) and the angiotensin converting enzyme (ACE) of hog kidney (17-19) will also be studied as the biochemistry of these

proteins has been studied extensively. The lower molecular weight of the lac transport protein (46 k Da vs. 95 k Da for Band 3 and ACE) may simplify the crystallization of this protein, although Band 3 and ACE are more typical of membrane proteins. Another integral membrane protein, bacteriorhodopsin, may also be used in the development of automated crystallization procedures as this protein is commercially available, and conditions for its crystallization, and its crystal structure, are already known.

A variety of chromatographic strategies, including hydrophobic interaction (HIC), ion-exchange (IEC), and reversed phase chromatography (RPC), will be tested for their utility in crystallization experiments. The milder chromatographic systems (i.e. HIC and IEC) have the advantage of maintaining the solubility of the protein without subjecting it to potentially denaturing conditions. Additionally, HIC is most similar to the phenyl-sepharose system previously described for 'chromatographic crystallization' (6) as the protein is applied to the column at high salt concentration and eluted from the column by slowly reducing the salt concentration. Although RPC is not generally applicable to the chromatography of membrane proteins, a solvent system employing detergent to increase solubility of the protein has been used successfully for the RPC of membrane proteins (8). It should also be noted that soluble enzymes have been purified by RPC with good recovery of activity. Detergent is often employed in the crystallization of membrane proteins (15,16), and may be beneficial in the crystallization of non-membrane proteins (A. McPherson, personal communication), and thus the addition of detergent to the solvent system may improve the chances of obtaining protein crystals. The recovery of proteins from HIC or IEC columns is generally quantitative even at the low sample loads proposed in this study (20), and the addition of detergent to the solvents used for the RPC of membrane proteins allows for quantitative recovery of protein over the range of 5 to 100 ug.

By using an HPLC mini-column, small samples of protein may be used for each experiment, with final protein concentrations of 5 mg/ml obtained when 500 ug of protein is eluted in 100 ul (a typical elution volume for a 4.6 mm inner diameter HPLC column). The use of narrow-bore columns, having an inner diameter of 2 mm, can further reduce the amount of sample required as the elution volumn would be about 20 ul. Further reductions in elution volumn could be achieved by using micro-bore column technology (i.e. columns with an inner diameter of 1 mm or less). Initially only the standard 4.6 mm column technology will be explored.

It is hoped that the strategies developed in our lab for automating the preparation of single crystals using laboratory robotics can also be applied to the automation of protein crystallization via this new chromatographic procedure such that a more rapid and efficient exploration of crystallization parameters can be routinely carried out.

3.1.3 Preliminary Results

HPLC mini-columns, aproximately 5mm x 10mm, were packed with hydrophobic interaction or wide-pore reversed-phase support. Chymotrypsin was chromatographed on the hydrophobic interaction column in ammonium sulfate and phosphate buffer using a reverse salt gradient. After determining appropriate chromatographic conditions, about 100 micrograms of chymotrpsin was injected onto the column and the eluted protein collected in six depression slides. These were covered with glass cover-slips and sealed with Dow Corning silicone grease. After 2 to 3 weeks, small crystals were observed in several of the slides.

3.2 CRYSTAL ANALYSIS

In the evaluation of crystals for crystallographic analysis, the shape and optical properties of crystals under the microscope are the 'primary' characteristics used in the selection of suitable single crystals for x-ray diffraction anaylsis. The ideal crystal would be 'chunky', having dimensions of 0.1 x 0.1 x 0.1 mm or greater, and exhibiting birefringence when examined under crossed polarizers. After selection and mounting of a suitable crystal, the diffraction pattern of the crystal is recorded. Occasionally, a supposedly suitable crystal will not diffract. In these cases, the crystallographer wonders if the crystal was not stable in th x-ray beam or if something other than protein was crystallized. A procedure was developed for analyzing these crystals by HPLC to determine crystal composition.

Crystals of microcystin, which did not diffract, were isolated from a hanging drop micro-vapor diffusion set-up. The crystals were washed with 'reservior' solution to remove any surface adsorbed protein and then dissolved in methanol. The dissolved crystals, wash solution and 'mother liquor' (from the micro-vapor diffusion set-up) were all analyzed by reversed-phase HPLC. A standard curve was prepared to correlate peak height to micrograms of microcystin injected. The results of this experiment showed that the crystals did contain microcystin. A more detailed analysis of the results showed that the crystals contained two isomers of microcystin in a ratio of 2.6:1; the wash and mother liquor contained only one isomer of microcystin, and the starting solution of microcystin contianed both isomers of microcystin in a ratio of 5.6:1. The large quantity of microcystin remaining in the mother-liquor indicated that the amount of microcystin was not a limiting factor in crystal growth. The fact that one isomer was totally depleted in this liquor suggested that the two isomers co-crystallized and when one isomer was depleted crystal growth stopped.

3.3 CRYSTALLOGRAPHIC TECHNIQUES USEFUL TO THE CHROMATOGRAPHER

During my tenure as a postdoctoral fellow at the Naval Research Laboratory, I had the opportunity both to contribute to the work of crystallographers, by ensuring that samples used in crystallization experiments were of well defined purity, and also to learn how proteins are handled by crystallographers during the crystallization process. As a result of this I have noted two techniques which are either not used, or are under-utillized during protein purification.

The simplest change for a biochemist to make in a purification procedure which could make a significant difference in recovery of native biologically active protein is to use polyethylene glycol as a precipitating agent in place of ammonium sulfate. Polyethylene glycol is routinely used by crystallographers to precipitate and crystallize proteins, and is preferred over ammonium sulfate in many cases as it is gentler.

Another problem during protein purification is exchanging a protein from one buffer solution into another. The biochemist purifying the protein will generally use dialysis, ultrafiltration, or size-exclusion chromatography for this. In the case where the new buffer is at a higher ionic strength than the starting buffer, this can also be done by borrowing a technique, i.e. vapor diffusion, from crystallographers. Using this technique a sample can be slowly moved from a low ionic strength solution into a higher ionic strength solution while at the same time concentrating the sample. In the case of shifting a sample intop a lower ionic strength solution vapor diffusion would not be useful aqs the sample volume would increase proportionately to the difference between initial and final ionic strength.

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CHAPTER 4

PROTEIN PURIFICATION - SUPPORT SERVICES

4.1 USE OF HPLC FOR ROUTINE SAMPLE SCREENING

The sucess or failure of crystallization experiments is often dependent on the purity of the sample. Therefore, all samples arriving at LSM were routinely screened using HPLC techniques to determine their purity. When neccessary, samples were also re-purified using HPLC techniques.

4.1.1 Adaptation Of 'classic' Methods

In many cases the adaptation of a 'classic' method to a modern HPLC method merely requires application of open column methods to an equivalent HPLC column. In this first step at adapting conditions, buffers and gradients are based on published separations. The chromatographic parameters in the HPLC separation are then adjusted to try to mimic the separation previously reported. This should not be confused with 'optimizing' the separation, as the goal at this point is to use HPLC methods to duplicate the earlier work. This allows easy identifcation of the separation products. After a separation has been achieved which can be easily compared to the original published separation, the emphasis shifts from duplicating prior work to improving upon it. This usually consists of optimizing the conditions for sample loading, and tuning the gradient to achieve the high resolution of the desired components. Often the separation is tuned by adjusting the buffer composition to account for differences between the HPLC and open column matricies.

4.1.2 Examples From The Lab

4.1.2.1 Cardiotoxin

New methods for the isolation and purification of elapid cardiotoxins have been evaluated over the past three years. The original purification of cardiotoxin used size-exclusion chromatography followed by ion-exchange chromatography on a carboxy-methyl cellulose column. Previously, investigators at Fort
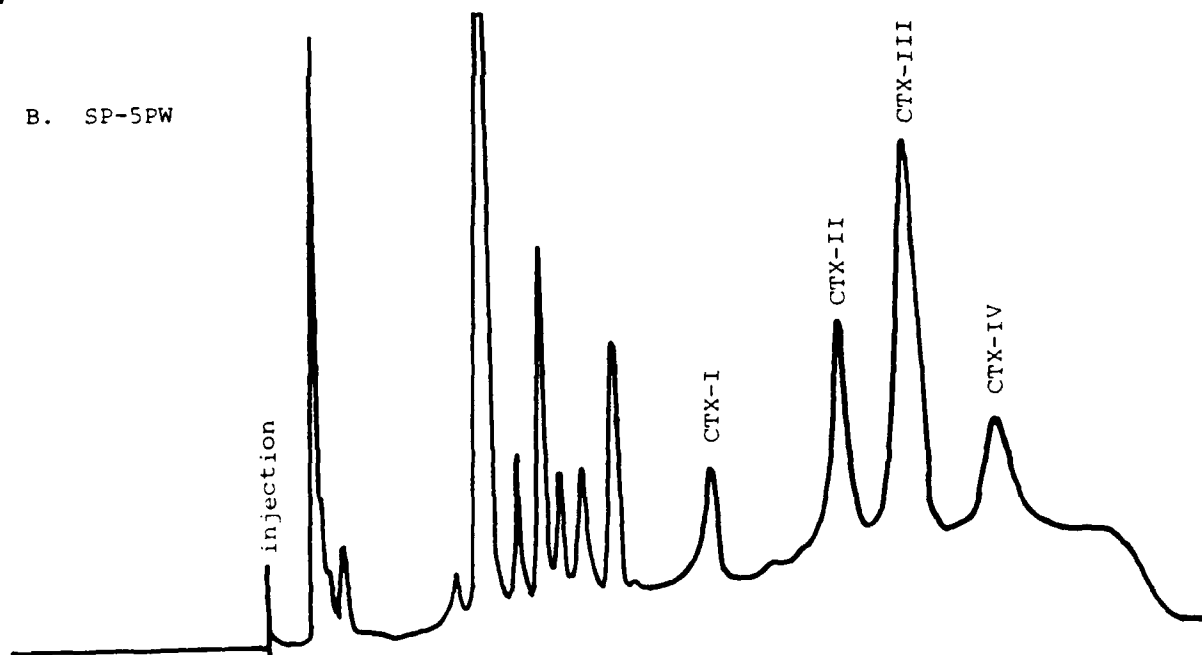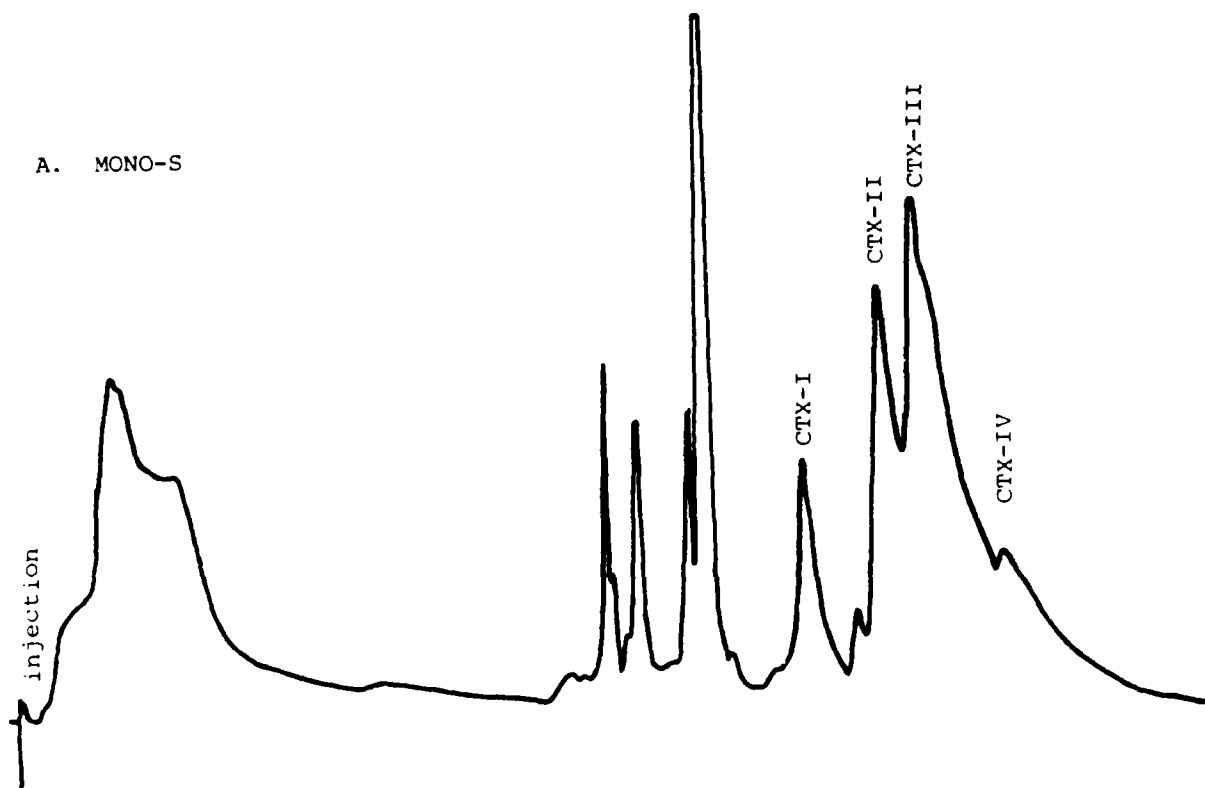
Detrich adapted the cardiotoxin purification to a 'fast-protein liquid chromatographic (FPLC) method using a 'mono-Q' column. Although this method offered higher resolution and shorter separation times than the classic open-column method, it suffered from poor peak shape, as did the open column method. In an attempt to improve the resolution of the various cardiotoxins, I have investigated other buffer systems and columns including cation-exchange and hydrophobic interaction columns with the following results:

1. Hydrophobic interaction chromatography, on a CAA-HIC column (Beckman Inst. Inc.), produced well resolved peaks with good symmetry at low sample loads, but cardiotoxins II and IV co-eluted; resolution was decreased at hicn sample loads.

2. Changes in buffer composition and gradient shape did have an effect on resolution, but were not effective in correcting peak asymmetry.

3. Chromatography of cardiotoxins from naja naja atra on the SP-5PW anion-exchange column (Toya Soda Inc.) produced excellent resolution and a dramatic improvement in peak shape (Figure 4.1). Resolution and peak shape were unaffected by sample load in the range of 50 micrograms to 40 milligrams, making this the method of choice both for screening samples of cardiotoxin for purity, and the purification of cardiotoxins on a semi-preparative scale (i.e. 10 to 40 mg).

The purification and crystallization provides an excellent example of the effect of sample purity on crystal growth. Sample of cardiotoxin were obtained from Dr. B.C. Wang's laboratory. Previously Dr. Wang had obtained cardiotoxin crystals from these same samples. His attempts at reproducing these crystals were unsuccessful. Sometime after the failed attempts at obtaining additional crystals the samples were sent to Dr. Ward at the Naval Research Lab. Using FPLC the purity of these samples was tested. None of the cardiotoxin samples from Dr. Wang's lab was homogenious. Cardiotoxins I and III were re-purified using FPLC. These re-purified cardiotoxins did produce crystals. Further experiments on these samples examined the effect of detergents on crystal growth.

FIGURE 4.1. Comparison of resolution and peak symetry of
             cardiotoxins chromatographed of different cation-
             exchange matricies. A. Pharmacia 'MONO-S' matrix
             shows significant peak asymmetry and poor resolution
             of cardiotoxins II to IV (i.e. CTX-II, CTX-II, and
             CTX-IV). B. Toya Soda 'SP-5PW' matrixs shows
             excelent resolution of all cardiotoxins and little
             or no peak asymmetry.

A. MONO-S

injection

CTX-I

CTX-II

CTX-III

CTX-IV

B. SP-5PW

injection

CTX-I

CTX-II

CTX-III

CTX-IV

4-4

4.1.2.2 Microcystin

Microcystin, a toxic cyclo-peptide, was chromatographed on C-8 and C-18 reversed-phase columns. Satisfactory retention was achieved only on the C-18 column. Using the following chromatographic conditions the sample was resolved into three components:

> solvent A - 5% ammonium acetate, pH 6.5
> solvent B - 5% ammonium acetate in methanol
> flow rate - 1.0 ml/min
> gradient program: isocratic at 0% B for 1 min, a linear
> gradient from 0 to 70% B in 10 min.

Using the acetate/methanol solvent system, three peaks were observed in the chromatogram. As the sample was reputed to be pure microcystin (i.e. >95%) it was thought that this may be an artifact induced by the chromatographic conditions. The chromatographic conditions were modified to more closely resemble the original chromatographic system; solvent A was changed to 0.1% trifluoroacetic acid and the experiments on the C-18 mini-column repeated. Under these conditions, three components were still detected, but the resolution was less than that observed in the 5% ammonium acetate. These three components have been consistently associated with microcystin, two of which are thought to be microcystin isomers, while the third, which exhibits significantly different chromatographic behavior, is thought to be a contaminant. Further investigation of these multiple peaks showed that resolution was pH dependent. Microcystin was purified using this method and the purified toxin used in crystallization experiments.

4.1.2.3 Aequorin

Aequorin is not homogeneous; it is known to consist of a number of isomers. Shimomura (Biochem J. (1986) 234: 271-277) has demonstrated that aeqourin can be fractionated into at least seven components using high performance ion-exchange chromatography (HP-IEC) on a Toya Soda DEAE-5PW column. Elution conditions consisted of a sodium acetate gradient in the presence of EDTA and 10 mM Mops (pH 7.1). The isolated aequorins were then charaterized by their UV absorbance spectra (and absorbance ratios at 455/280 and 205/280) and their luminescence.

The procedure descibed by Shimomura (1986) was modified to allow greater flexibility in the elution profile. This was accomplished by using distilled water for solvent A and 0.5M sodium acetate (pH 6.45) containing 2 mM EDTA for solvent B and adjusting the initial conditions such that the ionic strength was similar to the conditions used by Shimomura. The effect of calcium on the 'conformation' of aequorin was investigated by including 25 mM calcium chloride in the buffer and ommiting the EDTA. To discriminate betweeen ionic effects and conformational effects, the chromatographic behavior in the presence of calcium chloride was

compared to the chromatographic behavior in the presence of 50 mM potassium chloride (equal ionic strength to 25 mM calcium cloride).

Using the modified elution procedure, the eight previously reported isoforms of aequorin could be resolved. Of these, the six 'major' components were isolated, due to limited availibility of starting material only 0.1 to 0.2 mg of any individual isoform has been isolated. One of these components shows obvious signs of inhomogeneity, in agreement with the results of Shimomura. Additionally, it was found that long term storage of aequorin at -20 C (i.e. duration greater than one year) results in some degradation as evidenced by an increase in early eluting compounds during the ion-exchange chromatography, which do not emit light when exposed to calcium ions. Experiments in the presence of calcium provided preliminary evidence of a conformational change in aequorin after binding calcium. This was evidenced by a substantial decrease in retention in the presence of calcium that was not seen in the presence of an equal ionic strength of potassium. The presence of 50 mM potassium does not seem to alter retension significantly indicating no specific interaction (eg. ion pairing) between potassium and aequorin. The dramatic shift in retention in the presence of calcium indicates either a change in net charge on the aequorin or a change in conformation. These can be distinguished by comparing the chromatographic behavior in the presence and absence of calcium using a technique not sensitive to net charge (eg. hydrophobic interaction chromatography).

4.1.2.4 Staphylococcal Enterotoxin B

Staphylococcal enterotoxin B (SEB) lot P-50 was obtained from Fort Dietrich. Chromatography on a Mono-S column indicated that there were two major components and several minor components. The chromatographic conditions were optimized to improve the resolution of the various components, and the major components isolated using the following conditions:

    Buffer A: 5 mM sodium acetate (pH 5.6)
    Buffer B: 500 mM sodium actetate (pH 6.5)
    Flow rate: 1 ml/min
    Gradient: isocratic at 0% B, 1 min; linear gradient
                form 0% to 11.2% B, 15 min; linear gradient
                from 11.2 % to 80 % B, 4 min.

The fractions corresponding to the major components were pooled and desalted using an Amicon YM-2 ultrafiltration membranes.

CHAPTER 5

SUMMARY OF PRESENTATIONS

1988: The use of HPLC for Screening Fractions in Preparative
Chromatography: Application to cardiotoxin purification.
Ninth World Congress on Animal, Plant and Microbial
Toxins, Stillwater, OK.

1988: The purification and biochemical characterization of squid
hepatopancrease organophosphorous acid anhydrase. Second
OPA Anhydrase Workshop, Woodshole, MA.

1987: Protein Purification and Crystallization. Invitational
Seminar, Genex, Gaithersburg, MD.

1987: Application of hyper-purification to protein crystalliz-
ation. Mid-Atlantic Protein Crystallography Workshop,
NIDDK, NIH, Bethesda, MD.

1987: Rapid isolation of acetylcholinesterase (EC 3.1.1.7) from
snake venom. UCLA Symposia - Protein Purification: Micro to
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CHAPTER 6

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