DEVELOPMENT OF STRATEGIES FOR THE DOWNSTREAM PROCESSING OF ENZYMES/PROTEINS

By
IPSITA ROY
Chemistry Department

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However many words you read, however many you speak, what good will they do you if you do not act upon them?

- The Dhammapada
circa 200 B.C.

CERTIFICATE

This is to certify that the thesis entitled **DEVELOPMENT OF STRATEGIES FOR THE DOWNSTREAM PROCESSING OF ENZYMES/PROTEINS** being submitted by **Ms. IPSITA ROY** to the Indian Institute of Technology, Delhi for the award of the degree of Doctor of Philosophy in Chemistry is a record of bonafide research work carried out by her. Ms. Roy has worked under my guidance and supervision, and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma.

15th March 2001

M.N.Gupta
Professor & Head,
Department of Chemistry,
IIT Delhi.

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Ipsita Roy
(IPSITA ROY)

ABSTRACT

Downstream processing constitutes a major fraction of the production costs of enzymes/proteins. Thus, development of efficient and economical downstream processing strategies has today become an extremely important area in biochemistry/biotechnology. The trend is to develop protocols which have fewer steps, do not require any pre-processing and have unit processes which integrate well.

The present thesis largely focuses on further development of a downstream processing technique called expanded bed chromatography for protein purification. The last chapter also explores another emerging technique, viz. three phase partitioning.

The first chapter is an introductory chapter and briefly surveys the literature in the area of downstream processing in order to provide the necessary perspective for the work described in the thesis. A brief overview of the current downstream processing strategies is followed by a more focussed discussion on expanded bed chromatography and three phase partitioning.

One disadvantage of using commercially available media for expanded bed chromatography is their high cost. Hence it was thought worthwhile

to attempt the purification of cellulase from a commercially available fungal source on an expanded bed of indigenously developed cellulose beads. This is described in Chapter II.

The conditions for binding of cellulase to cellulose beads and the elution of the bound activity were first tried in the batch mode. Under optimum conditions, 85 % of the loaded activity could be bound to the beads. Out of the various eluents tried, 1 M sodium phosphate buffer, pH 7.0 was found to recover the bound activity almost quantitatively.

Purification of cellulase was then attempted in a packed bed of cellulose beads. Elution with 1 M sodium phosphate buffer, pH 7.0 gave a recovery of 82 % of the initially loaded activity. The enzyme was purified 15-fold.

The beads were then evaluated for use as matrix for expanded bed chromatography by studying bed expansion as a function of the liquid flow rate. The data showed that the adsorbent was suitable for use as a matrix for expanded bed chromatography. Nearly all the loaded activity could be bound in the expanded bed mode. Elution in the packed bed mode succeeded in recovering 91 % of the enzyme activity. The enzyme was purified 30-fold. SDS-PAGE of the purified sample showed a single band.

The work described in Chapter III aims to use chromatographic media made from polysaccharides for affinity capture of α-amylases and cellulase from crude extracts. Conjugation of an affinity ligand to a

polymeric support, with its inherent problem of leaching of the bound ligand, is thus not required.

The purification of α-amylases from four different sources, viz. porcine pancreatic, bacterial (*Bacillus amyloliquefaciens*), wheat germ and fungal (*Scytalidium thermophilum*) was tried on a fluidized bed of alginate beads. Adsorption isotherms of amylases on alginate beads showed that the enzyme from the bacterial source and porcine pancreas bound equally well with alginate beads of low as well as high mannuronic acid content whereas the enzyme from wheat germ extract and the fungal source showed preference for beads prepared from alginate with high mannuronic acid content.

Both kinds of beads were tested for use as media for fluidized bed chromatography. Bed expansion results showed that they fit well with the Richardson-Zaki relation. The enzymes from the four sources were purified on fluidized beds of alginate beads. The bound enzymes could be recovered from the column with high yields. The wheat germ enzyme was purified 58-fold. The considerable purification achieved is reflected in the SDS-PAGE pattern of the purified enzyme.

The enzyme from the fungal source could be purified 9-fold with an activity recovery of 80 %. The thermal stability of the purified enzyme (E I) was determined at 80 °C with and without the addition of Ca^{2+} ions. In both cases, the enzyme lost two-thirds of its activity almost immediately at this temperature. However, in the presence of Ca^{2+}, the

remaining activity was quite stable up to 3 h. Assuming first order thermoinactivation kinetics, the half-life ($t_{1/2}$) for this surviving activity, in the presence of Ca^{2+}, worked out to be 38 h. This behaviour suggested that there might be two isoenzymes with similar molecular weights but with different thermostabilities. As fungal amylases are known to be glycoproteins, the purified enzyme was passed through a Con A-agarose column. About one-third of the activity bound to the column and it could be quantitatively eluted (E III) with 0.01M α-methylmannopyranoside. The thermal stability of the two fractions, viz. the one which remained unbound to the column (E II) and the fraction that eluted from Con A-agarose column (E III) and were re-examined at 80 °C in the presence of Ca^{2+} ions. In both cases, exposure to 80 °C in the presence of Ca^{2+}, led to partial inactivation of the enzyme and only 33 % of the original activity survived till 10 min. In the case of E II, further exposure led to rapid inactivation of the enzyme. E III, on the other hand, did not lose any further activity, even up to 3 h. The results clearly indicate that we are dealing with (at least) two isoenzymes.

Chitosan beads were next prepared for use as matrix for affinity capture of fungal cellulases. Adsorption isotherms of cellulases (from two different fungal sources, viz. *Aspergillus niger* and *Aspergillus fumigatus*) on chitosan beads were drawn. Because of its higher affinity for chitosan beads (as deduced from the adsorption isotherms), the cellulase from *Aspergillus niger* was selected for purification purposes.

Conditions of binding and elution of the bound enzyme were optimized in the batch mode. Purification of the bound enzyme was then attempted on a fluidized bed of chitosan beads. About 80 % activity recovery with 30-fold purification in a single step showed that this may also be a useful approach for purification of cellulases. The purified enzyme was found to be free of β-glucosidase activity and in fact, showed a single band on SDS-PAGE.

These results thus indicate that screening the binding of enzymes to various naturally occurring polysaccharides may lead to some interesting possibilities in affinity-based separation techniques.

The work described in Chapter IV focuses on the purification of alkaline phosphatase from chicken intestine using a single step of expanded bed affinity chromatography by utilizing Cibacron Blue dye linked to cellulose beads.

Seven dyes were linked to cellulose beads and screened for binding alkaline phosphatase under different conditions. The two dyes which showed maximum binding of the enzyme, viz. Cibacron Blue and Navy HER, were chosen for purification of the enzyme in the batch mode. The recovery of the bound enzyme from these two matrices was optimized by varying the eluting salt concentration.

When efforts were made to purify the crude enzyme on Navy HER-linked cellulose beads in the expanded bed format, about 72 % activity bound to the column. However, no enzyme activity could be eluted from

the column with the eluents optimized in the batch mode. In the system described in Chapter II too, we have observed that the results obtained in the batch mode do not translate into the expanded bed mode.

The Cibacron-Blue linked cellulose beads however, could be successfully used in the expanded bed mode for the purification of the enzyme. The enzyme could be purified 48-fold with 70 % recovery of the enzyme activity.

In Chapter V, a simple two-step purification protocol for a polyphenol oxidase (PPO) isoenzyme from *Duranta plumieri* seeds by using expanded bed chromatography on a commercially available ion exchanger (Streamline^{TM} DEAE) followed by use of Con A-agarose (in the batch mode) is described.

The dynamic binding capacity of the adsorbent for the enzyme was found to be only slightly less than the equilibrium binding capacity determined from the adsorption isotherm experiments. Purification of the enzyme from the crude extract by expanded bed chromatography resulted in the recovery of 77 % of the enzyme activity. This major fraction of the activity showed three bands on SDS-PAGE. This fraction was further treated with Con A-agarose in the batch mode. About 60 % of the loaded enzyme activity bound to Con A-agarose. About 50 % of the bound activity could be eluted off the beads by 0.01 M α-methylmannoside. This purified enzyme showed a single band on SDS-PAGE, when stained with Coomassie Blue. This isoenzyme was further

characterized in terms of molecular weight, kinetic parameters and thermal stability wherein it was shown to exhibit the phenomenon of "latency" or "activity regeneration".

The second part of the chapter deals with the purification of a 'double-headed' α-amylase / Proteinase K inhibitor from wheat germ on another commercially available matrix, viz. Streamline^{TM}-chelating.

The endogenous α-amylase could be precipitated by heating at 70 °C. The resin was loaded with Cu (II) and used for the purification of the inhibitor in both packed and expanded bed modes. Both the procedures yielded similar fold purification and enzyme recovery. However, in the case of expanded bed mode, it was not found necessary to separate the precipitated amylase (and other protein impurities) before the chromatographic run. The purified inhibitor showed a single band on SDS-PAGE with an apparent molecular weight of 21 kDa and was shown to inhibit Proteinase K activity as well as α-amylase activity.

Chapter VI deals with protein-solvent interactions involved in another downstream processing technique, viz. three-phase partitioning. In this technique, ammonium sulphate and an organic solvent (normally t-butanol) are used to precipitate enzymes at the interface of the two layers, from an aqueous solution.

One of the main assumptions attributed to the success of this technique has been that t-butanol binds to the interior of the protein. Our results with Proteinase K have shown that this binding is not the cause of either

the enzyme being forced out of the aqueous phase or the remarkably enhanced biological activity observed (210 % increase in specific activity) after the enzyme has been subjected to three phase partitioning. X-ray diffraction studies indicated that the enzyme occurs in a more flexible state, which presumably is responsible for the higher turnover number.

It was also shown that metal affinity interfaced with TPP makes the latter process more selective. The system chosen as the "proof of concept" was soybean trypsin inhibitor.

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