OPTIMIZATION OF SAMPLE PRETREATMENT METHODS FOR THE APPLICATIONS OF THE GLUTAMATE OXIDASE REACTOR IN FLOW INJECTION ANALYSIS OF THE NEUROTOXIC β-ODAP IN GRASS PEA *(guciyci)*

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KASSBVI AHMED

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DEDICATION

To my Wife, my Children and my Parents

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OPTIMIZATION OF SAMPLE PRETREATMENT METHODS FOR THE APPLICATIONS OF GLUTAMATE OXIDASE REACTOR IN FLOW INJECTION ANALYSIS OF THE NEUROTOXIC β-ODAP IN GRASS PEA *(guaya)*

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ABSTRACT

In the determination of β-ODAP by flow injection analysis using a glutamate oxidase reactor, the major problem is that proteins and other macromolecules cause a rapid decay of the activity of the reactor when an extract of grass pea is directly injected into the reactor.

In this work, off-line and on-line protein separation is described. In a preliminary study, a standard protein sample, bovine serum albumin (BSA), was treated with acidic alumina at different pH. Excess BSA was washed and the absorbance of the supernatant was taken at 280 nm. It was found that 84% of the protein was adsorbed on alumina at pH 7 (off-line separation). The recovery of β-ODAP was also studied at different pH by treating it with the alumina. Absorbance at 220 nm, before and after its treatment with the alumina revealed 100±5% recovery at pH 7.

BSA and β-ODAP were combined and treated with alumina to check whether their coexistence has an effect on their interaction with alumina. Since β-ODAP may interfere with the measurement carried at 280 nm, the amount of the adsorbed protein was quantitated at 750 nm (Lowry-Folin method) and found to be 85% which is in a good agreement with the result obtained when the measurement of BSA was carried alone (84%).

The strong anion exchanger (SAX), trimethylaminopropyl Isolute SAX(500-0010A), was employed for on-line separation of proteins. Of the injected standard protein and those from extracts of grass pea, 98% were retained by the sample clean-up column at a pH of 6.5 . This indicates that effective removal of proteins was achieved. The sample clean-up column has no significant effect in retaining β-ODAP and 98% of the injected standard β-ODAP was recovered.

1.1. Distribution and Nutritional Value of LS

The legume, *Lctthyrus Scttivus* (also known as *guayci* in Amharic), is widely cultivated in areas where farmers are dependent on rain-fed agriculture like Ethiopia, Bangladesh, India, Syria, China and Nepal. The plant is cultivated to the extent that it occupies significant proportion of the pulse produced in these areas (e.g. Ethiopia 7.5%, Bangladesh 34%) [6]. The reasons for the wide cultivation of the legume are: (1) economically, it is the cheapest legume available; (2) it is tolerant to any weather requiring very little agricultural attention and is considered as a life-saviour in times of drought and famine.

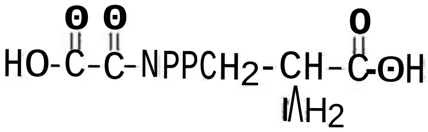
The edible seed contains about 28.2% protein, 58.2% carbohydrate, 0.6% fat and 3% mineral matter [7]. It has, therefore, been an important alternative source of nutrition for human consumption and fodder for live-stocks.

1.2. Neurolathyrism

Lathyrism is a disease which causes an irreversible paralysis of human legs. In 1957 Hans Selye proposed the term Neurolathyrism to describe the syndrome which affects the nervous system of man (human lathyrism) [8]. Neurolathyrism is characterized by symptoms such as muscular rigidity, weakness and paralysis of the leg muscles, convulsions, head retraction, stiffening of the neck and death in extreme cases. The toxicity of LS seeds has been known in the western and eastern world since historical times. The disease reaches epidemic proportion in times of famine caused by flood and drought. Relatively recently, outbreaks of lathyrism occurred in Ethiopia and Bangladesh [9]. In human, continuous consumption of two-third to one part of their diet for two or three months was considered enough to cause a disease, although not all persons eating the diet seemed to be affected; in families only certain members were attacked. Males were said to be more susceptible than females [10].

1.3. The Toxic Principle

The non-essential amino acid, β-ODAP was identified 30 years ago by two independent research groups in India [1] to be the constituent responsible for human lathyrism. This non-essential amino acid has been detected commonly in LS and other species. The chemical structure of the toxic compound, β-ODAP or its synonym (β-N-oxalyl amino L-alanine, BOAA) is :



$$HO-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{C}}-NPPCH_2-\underset{NH_2}{CH}-\overset{O}{\overset{\|}{C}}-OH$$

The compound is found to be a powerful convulsant [1] and is neuroexcitatory to central nervous system neurones. In the plant, it exists in two forms: α and β, where the α-isomer is to the extent of approximately *5%* and is found to be non-toxic or of low toxicity [12].

Bell and O' Donovan reported [13] that in ethanolic solution β-ODAP slowly equilibrates with the α-isomer and the interconversion is facilitated when heat is involved. This observation and the non-toxicity of the α-isomer paves a way to explore various processing and cooking methods as a means for the thermal detoxification of the legume.

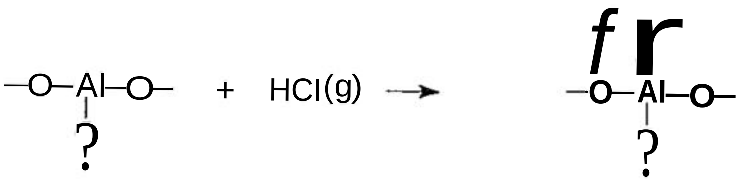
1.4. Analytical methods for the determination of β-ODAP

Researchers in several institutes are developing methods to find a species of a grass pea with no or low toxin. It is anticipated that plant breeding and genetic engineering [14] may be used to achieve this goal. But this should be accompanied by developing a fast and selective method for monitoring the neurotoxin from a large number of samples. Analytical methods for the determination of β-ODAP can be distinguished as enzymatic and non-enzymatic methods.

Non-enzymatic methods are non-selective and experimentally inconvenient [15]. The most widely used non-enzymatic method is the Rao method. In this method the reaction of o-phthalaldehyde (OPT) with L-α, β-diaminopropionic acid, the product of alkaline hydrolysis of both *α*- and β-isomers of ODAP, is monitored at 420 nm [2]. This methods has the following limitations: (1) it requires 6 h extraction and 1 h sample pretreatment prior to the final determination; (2) it lacks selectivity since the a-isomer hydrolyses to diaminopropinoic acid (DAP) and is detected together with β-ODAP. The need for a selective method for the determination of β-ODAP is therefore obvious.

The enzymatic method which involves the enzyme DAP-ammonia lyase for the determination of ODAP after alkaline hydrolysis to DAP was equally unselective as the Rao method [16]. Recently it was found that the enzyme glutamate oxidase (from streptomyses, SP) catalyses the oxidation of β-ODAP selectively [3]. The oxidation product, hydrogen peroxide and ammonia are then detected. In this method, an immobilized GLOD reactor is used in a flow injection analysis (FLA) of β-ODAP. The new method is evidently an important progress towords β-ODAP analysis in monitoring a large number of samples from the field. However, an effective separation of proteins from LS seed extracts has to be developed since adsorption of proteins on the enzyme reactor is the major disadvantage behind the newly reported flow injection analysis of ODAP. In the method reported [20] protein separation was performed by ultra-filtration method which is not commonly available in laboratories of the countries severely affected by lathyrism. Thus, methods of protein separation have to be optimized to circumvent the problem.

The objective of this work is therefore to optimize methods of protein separation for glutamate oxidase (GLOD)-FI system using acidic alumina and strong anion exchanger.



Ion-exchange chromatography is a variation of adsorption chromatography in which the solid adsorbent has charged groups chemically linked to an inert solid. Ions are electrostatically bound to the charged groups; these ions may be exchanged for ions in an aqueous solution. Ion-exchangers are most frequently used in columns to separate molecules according to charges that they bear. Since charged molecules bind to ion-exchanger reversibly, molecules can be bound or eluted by changing the ionic strength or pH of the eluting solvent.

Three general methods are used for eluting molecules from the exchanger: (a) changing the pH of the buffer to a value at which binding is weakened (i.e. the pH is lowered for an anion-exchanger and raised for a cation-exchanger), (b) increasing the ionic strength by increasing the concentration of salt in the elution solvent, thereby weakening the electrostatic interaction between the adsorbed molecule and the exchanger, and (c) performing affinity elution. In affinity elution the adsorbed molecule is usually a macromolecule that is desorbed from the affinity ligand by adding a molecule that is charged and of opposite sign to the net charge on the macromolecule. Thus, the reduction of the net charge on the macromolecule weakens its electrostatic interaction with the exchanger sufficiently to permit the elution of macromolecule from the affinity ligand [24].

Ion-exchange chromatography is probably one of the most widely used procedure in purifying proteins [24-26]. Several hundred proteins have at least one step in their purifications, that is an ion-exchange procedure. The anion-exchangers are more commonly employed than are the cation exchangers because there are more acidic proteins than there are basic proteins. In this work on-line and off-line separation of proteins was investigated by using the strong anion-exchanger (SAX) and alumina, respectively. Elution of the proteins from SAX was achieved by changing the ionic strength of the buffer.

Medium dispersion is chosen when mixing and reaction of the analyte with the reagent is required to form a detectable product. Partial mixing between the injected sample zone and the reagent can be affected by using long tubes, low flow rate, and several channels with various mixing points.

Large dispersion is employed only when the sample should be diluted in order to determine its concentration in a linear range of analysis. In such systems where a very long reactor is used, the residence time is very long.

The easiest way of measuring the dispersion coefficient is to inject a well-defined volume of a dye solution into a colourless carrier stream and to monitor the absorbance of the dispersed dye zone continuously by a colorimeter. The Devalue is obtained by measuring the height of the recorded peak for the dispersed dye and comparing it with the distance obtained between the baseline and the signal monitored when the cell has been filled with undiluted dye. The ratio of the respective absorbance gives a Dm" value that describes the FIA manifold, detector and methods of detection, provided that Lambert-Beer law is obeyed [40]. Factors which affect the degree of dispersion and, therefore, the recorded peak hight are: injected sample volume, flow rate, channel length and geometry.

Changing the injected volume is a powerful way to change dispersion. An increase in peak height and in sensitivity of measurement is achieved by increasing the volume of the injected sample solution. Conversely, dilution of highly concentrated sample material is successfully achieved by reducing the injected volume.

The dispersion of the sample zone increases with the square root of the distance travelled through the tubular conduit and decreases with decreasing flow rate. Thus, if dispersion is to be reduced and the residence time to be increased, the tube dimensions should be minimized and the pumping rate should be decreased. The most effective way to increase the residence time and to avoid farther dispersion is to inject the sample into a flowing stream and stop the stream's forward movement, then resuming pumping after a sufficient reaction time has elapsed [36, 47].

Different channel geometries are used in FIA. These are straight tube, coiled tube, mixing chamber, single-bead string reactor, 3-D or "knitted" reactor, and imprinted meander or combination of these geometries. The function of these reactors is to increase the intensity of radial mixing, by which the parabolic velocity profile in the axial direction formed when the sample zone is injected into a laminar flow of carrier stream, is reduced [36]. A coiled tube can conveniently accommodate any length of tubing in an experimental set up and secondary flow within it promotes radial mixing. Therefore, it is the most frequently used reactor geometry [36].

Since its conception, FIA has grown rapidly. Nowadays, many believe that it has the potential to become the method of choice for many automated and semiautomated assays. The advantages of FIA as compared to SFA are: (1) its simplicity, (2) higher sampling frequency, (about 100 samples /h), (3) the lower consumption of sample and reagents, and (4) flexibility of introducing all the necessary reagents into the carrier.

One of the great advantages of the FIA technology is the ease with which additional component can be added to the system to achieve a particular analytical objective. In addition to sample assays, employing one or several reagents, FIA system may be designed to dilute or to concentrate the analyte; to perform separation based on ion exchange, gas diffusion or dialysis; to generate unstable reagents to the concentration suitable for a given assay [36-37, 41]. Ion exchangers, for example, have been employed as a column materials in order to preconcentrate an analyte, or to remove matrix components that might interfere, or to convert a sample constituent into a detectable species [37].

2.6. Applications of Immobilized Enzyme Reactors(IMERs) in FIA

Enzyme reactors have found applications in the analysis of species of biomedical, biochemical, food and agricultural interest. In FIA they can be applied for the determination of single analyte, multi analyte and for the elimination of interference. In single analyte determination, the analyte is allowed to react selectively with a coenzyme or coreactant to yield detectable species which then is carried into flow cell to be measured.

In multi-analyte determination a sample is divided into different portions which are monitored simultaneously or sequentially. For example, simultaneous determination of two components in a sample can be achieved by making the carrier to flow in two directions so that one portion flows through both reactors and the other only through the second reactor. A portion which passes through the first reactor is delayed by a delay coil to enable the other portion to reach the second reactor before it. Thus, two signals will be obtained in succession from the detector [39]. In the case of sequential determination, a bypass which is controlled by a switch valve is made around the first reactor. The valve enables the flow to pass either through both reactors (for the determination of both components) or through the second reactor only (for the determination of a single component). Thus, two sample injections allow both components to be determined in this system.

Interfering species, which may contribute to the detected signal with or without being involved in the reaction occurred in analytical or indicator reactor, can be destroyed by using an appropriate pre-reactor [42]. Similarly, if the reagent stream contains a substrate which behaves similarly as the analyte, insertion of a reactor along the reagent channel may also destroy the interference [43].

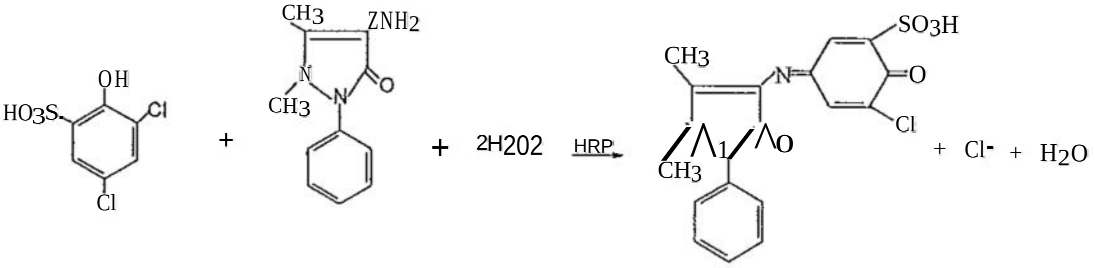
If the enzyme in the pre-reactor is very active to one of the substrate in the sample and much less active to the other substrate, selectivity to the latter can be achieved by using a small reactor so that the substrate to which it is less active (substrate to which selectivity is sought for) will not be destroyed [20].

2.7. Determination of Hydrogen Peroxide

Hydrogen peroxide is one of the products produced in the reaction catalysed by a class of enzymes called oxidases. For example, the oxidation products of glucose catalysed by glucose oxidase are hydrogen peroxide and gluconic acid. The hydrogen peroxide is directly proportional to the concentration of the substrate. Thus, if some means is devised to monitor hydrogen peroxide, the concentration of the substrate can indirectly be determined. There are several techniques to monitor hydrogen peroxide that have found applications in diverse analytical situations.

These techniques have been described in papers found in the literature [44] and range from those designed for industrial applications to clinical assays employed in monitoring the composition of body fluids. However, in dealing with peroxides of low concentration only few of this techniques have been suitable or reliable. Among these few, peroxidase-coupled oxidation of donor substrate systems has gained popularity.

HRP has been used in conjunction with Trinder's reagent (2,4-dichlorophenol-6-sulphonate, DCPS and 4-aminophenazone, 4-AP) for FI determination of hydrogen peroxide. The possible chromogenic reaction is:



(2.7.5)

Glutamate oxidase catalyzed reaction of the neuro-toxic β-ODAP was monitored *via* Trinder's reaction (equation 2.7.5) for assaying the toxin in grass pea extracts after its oxidation in a GLOD reactor [16, 44]. In the oxidation reaction a-keto acid (β-N-oxalyl-a-keto-β-aminopropionic acid), ammonia and hydrogen peroxide are produced (equation 2.7.6) [16].

$$\mathrm{HOOC}\underset{\underset{\displaystyle \mathrm{O}}{\|}}{\mathrm{C}}\mathrm{NHCH_2CHNH_2COOH} + \mathrm{H_2O} + \mathrm{O_2} \xrightarrow{\mathrm{GLOD}} \mathrm{HOOC}\underset{\underset{\displaystyle \mathrm{O}}{\|}}{\mathrm{C}}\mathrm{NHCH_2}\underset{\underset{\displaystyle \mathrm{O}}{\|}}{\mathrm{C}}\mathrm{COOH} + \mathrm{H_2O_2} + \mathrm{NH_3} \qquad (2.7.6)$$

Of the products hydrogen peroxide is coupled *via* Trinder reaction in a similar manner as in the above cases and is monitored at 512 nm. The content of β-ODAP in a grass pea is thus determined using the 1:1 stoichiometric relationship of hydrogen peroxide and β-ODAP (equation 2.7.6).

3. EXPERIMENTAL

3.1. Chemicals and Reagents

D-(+) glucose (Sigma), L-glutamic acid (Sigma), 4-aminophenazone (4-AP) (BDH), 2,4-dichlorophenol-6-sulphonate (DCPS) and acidic alumina (mesh 70-230, particle size 0.065-0.200 mm, Merck) were used as received. Bovine serum albumin (BSA) was kindly supplied by the Department of Biology (AAU). β-ODAP was synthesised by the Rao method [59]. Hydrogen peroxide was standardized by titrating with oxalate-standardized permanganate [48].

The reagent for the FI system was prepared in 0.1 M phosphate buffer, pH 7, and consisted of 2.5 mM DCPS, 0.5 mM 4-AP, 0.5 mM EDTA and 0.02 mg/mL horse radish peroxidase (HRP, E.C. 1.11.1.7, 290 purpurogallin U/mg solid, Sigma). The carrier in the flow system was 0.1 M phosphate buffer (pH 7) unless otherwise specified.

The Lowry protein reagent consisted of reagent A: 0.5 g copper sulphate and 1 g sodium citrate dissolved in 100 mL of water; reagent B: 20 g sodium carbonate and 4 g sodium hydroxide dissolved in 1 litre of water; reagent C: 50 mL of reagent B mixed with 1 mL of reagent A; and reagent D: 1 : 1 diluted Folin-Cicalteau reagent (Sigma) [43, 49].

3.2. Protein Separation

Preliminary study

0.1 mL of 10 mM β-ODAP solution was added to one series of buffer solution pH 2 to 7 with a volume of 1.9 mL each. 0.1 mL of 3 mg/mLof BSA was added to the other series of buffer solution of the same pH and volume. To both series 100 mg acidic alumina was added and kept for about 2 h. After 2 h, both series of solutions were centrifuged and finally filtered by washing the alumina throughly with 3 mL of distilled water and the filtrates of both series were taken for absorbance measurements at 220 and 280 nm respectively, using Milton Roy spectrophotometer. The absorbance of the same series of solutions was also taken without

3.3. Immobilization of Enzymes

300 mg of controlled pore glass (CPG-10 with 0.12-0.2 mm particle size and 51 mm pore size, Serva) was treated with concentrated nitric acid and silanized according to earlier reported procedure [20]. The silanization was tested by adding 3 drops of 1.5% 2,4,6-trinitrobenzene sulphonate solution in ethanol into a test tube containing small amount of the derivatized CPG mixed with saturated sodium borate solution. A deep orange colour appeared, confirming a positive test [53]. The silanized support was activated for 45 min at reduced pressure with 5% glutaraldehyde in 0.1 M phosphate buffer at pH 7. The activated support was thoroughly washed with distilled water over a G-3 filter. Prior to activation, the stock 25% glutaraldehyde solution (Sigma, G-6257) was mixed with activated charcoal and centrifuged to remove any possible polymeric product.

6 mg of glutamate oxidase, (GLOD, E.C. 1.4.3.11, 6.8 Unit/mg from Yamsa corp., Japan) was dissolved in 4 mL of 0.1 M phosphate buffer at pH 7. The enzyme in the solution was immobilized on 180 mg of the glutaraldehyde-activated CPG support at reduced pressure for 45 minutes. The immobilized enzyme support was packed in 150 μL plexiglass tube for flow injection application and stored at 4 ^{0} C when not in use.

Following the same procedure, 1 mg and 10 mg of glucose oxidase (GOD, Penicillium notatum, E.C. 1.1.3.4, 767 mmol/s protein, Veblca-Feinchmie Sebnitz) were also immobilized on 10 mg and 100 mg of the activated support respectively.

3.4 Flow Injection Set Up

The assembly of the FI system for β-ODAP assay is basically the same as that reported elsewhere [20, 52] except that an SAX-packed plexiglass tube was inserted for on-line protein separation. As in the previous reports, the system consisted of a two channel peristaltic pump, P (Gilson , model M312), an injection port, S (Rheodyne injection valve), SAX packed plexiglass tube, analytical reactor (GLOD reactor), a mixing tee, M, a coiled tube for effective mixing, C, a flow- through detector, D (LKB 2151 UV-Vis spectrophotometer), a recorder, R (Pederson Strip Chart recorder Model 27MR, California) and a tube to remove waste (W). The flow rate of the sample line was 0.31 mL/min and that of the reagent line was 0.11 mL/min when 150 μL plexiglass tube was assembled.

To test reactor stability and to check effectiveness of the sample pretreatment by an off-line separation the GLOD reactor in the set up was replaced by 150 and 50 μL GOD reactor and the sample clean-up was not used (Fig 3.1). For the study of an on-line sample pretreatment in the absence of GLOD reactor, the set up was used without reagent line.

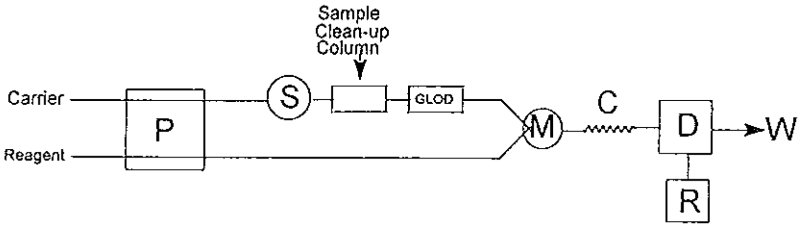


Fig. 3.1. Flow injection set up.

3.5. Extraction of ODAP from Grass pea

For the sample pretreatment with acidic alumina and SAX, β-ODAP was extracted from 100 mg of grass pea in 10 mL of 0.1 M phosphate buffer pH 7 in the former and 0.02 M phosphate buffer of the same pH in the latter case. The extraction was effected over ice water bath by agitation with magnetic stirrer for 2 h [20, 43]. Particulate matters were removed by centrifugation and filtration through a 0.45 μm membrane filter.

4. RESULTS AND DISCUSSION

4.1. Optimization of the FI System

Flow rate is inversely proportional to the residence time of the substrates and the products within the enzyme reactor [53]. Thus, decreasing the flow rate will increase the conversion efficiency of the reactor. There is an optimum flow rate required for equilibrium to be reached, depending on the activity of the enzyme in a reactor. The effect of flow rate was studied by injecting 20 μL of 30 μM standard β-ODAP in the presence of 150 μL GLOD reactor. Maximum peak height was obtained at a total flow rate of 0.31 mL/min, but sampling frequency was only 12 samples/h. Therefore, studies on calibration, reactor stability and investigations on removal of proteins by off-line sample pretreatment were made at a total flow rate of 0.41 mL/min. In the presence of sample clean-up column (for on-line sample pretreatment) and enzyme reactor the total flow rate was 0.42 mL/min. The optimum flow rate for the sample line (0.295 mL/min) and reagent line (0.115 mL/min) were obtained by using pump tubes of 0.6 and 0.23 cm^2/m, respectively.

The optimum pH and reagent concentration for GLOD reactor, in the absence of sample clean-up column were the same as reported before [20]. When sample clean-up column was assembled , 0.02 M phosphate buffer, pH 6.5 was used but reagent concentration was the same.

4.2. Calibration Curve

Standared hydrogen peroxide, L-glutamic acid and β-ODAP were calibrated using, in all cases, the chromogenic reaction of H_2O_2 with Trinder's reagent as a basis for calibration . The absorbance of the product of this chromogenic reaction when (a) pure H_2O_2 (b) glutamic acid and (c) β-ODAP are injected and made to pass through the reactor, was measured as a function of concentration. The calibration curve was linear in the range 1-400 μM for hydrogen peroxide and glutamic acid but only 10-50 μM for β-ODAP (R=0.998).

4.3. Optimization of Methods of Protein Separation

It was reported [20] that the response of the FI-GLOD reactor for β-ODAP determination became erratic and unreliable after direct injection of a number of crude LS extracts were made into the reactor. This was attributed to the adsorption and accumulation of cell fragments on the surface of the solid biocatalyst. In the reported system proteins and other macro-molecules were removed by off-line ultra-filtration [20]. Thus, sample pre-treatment before injection becomes necessary in order to obtain reliable experimental data and to prolong the life time of the GLOD reactor. Ultra-filtration membranes are not commonly available and therefore, other options had to be investigated.

In the preliminary study of protein separation the pH at which adsorption of protein on alumina and recovery of β-ODAP is maximum was studied by treating both BSA and β-ODAP at different pH (Table. 4.1 and 4.2).

Table.4.1. Absorbance of BSA at λ_{max}= 280 nm before and after addition of alumina as sorbent at different pH.

pH of BSA solution	Absorbance before treatment	Absorbance after treatment	% Adsorbed
2	0.403	0.073	82±0.40
3	0.369	0.076	79±0.21
4	0.391	0.034	91±0.23
5	0.382	0.040	89.5±0.30
6	0.358	0.042	88.3±0.25
7	0.375	0.057	84± 0.45

Table 4.2. Absorbance of β-ODAP at λ_{max} = 220 nm before and after addition of alumina as sorbent at different pH.

pH of β-ODAP solution	Absorbance before treatment	Absorbance after treatment	% Recovery
2	0.262	0.060	23±0.21
3	0.167	0.064	38.3±0.15
4	0.292	0.136	46.6±0.02
5	0.272	0.071	26±0.18
6	0.338	0.234	69.2±0.11
7	0.328	0.325	100±5

The preliminary study on protein separation by using acidic alumina as sorbent indicated that 84% BSA, taken as a model, was adsorbed and 100±5% β-ODAP was recovered at pH 7 (Table. 4.3).

Table 4.3. Summary of protein separation and recovery of β-ODAP by treatment with 100 mg alumina at different pH. λ_{max} = 280 nm for BSA and 220 nm for β-ODAP.

pH of the solution	% protein (BSA) adsorbed	% β-ODAP Recovery
2	82±0.40	23±0.21
3	79±0.21	38.3±0.15
4	91±0.21	46.6±0.02
5	89.5±0.30	26±0.18
6	88.3±0.25	69.2 ±0.11
7	84±0.45	100 ±5

The adsorption behavior when both BSA andβ-ODAP exist together was also studied. However, in the determination of BSA its absorbance at 280 nm was subjected to an interference from β-ODAP. This problem was circumvented by determining BSA at 750 nm (Lowry reaction, Sec 3.1). The result obtained (85%) was in agreement with the one determined in the absence of β-ODAP, indicating that their coexistence has no effect on their interaction with alumina (Table. 4.4).

Table 4.4. Comparison of the percentage of BSA adsorbed in the presence and absence of β-ODAP.

Absorbance at 750 nm, before treatment with alumina	Absorbance at 750 nm, after treatment with alumina	% BSA adsorbed, as determined at 750 nm, by Lowry method in the presence of β-ODAP	% BSA adsorbed, as determined at 280 nm, in the absence of β-ODAP
0.333	0.05	85±0.15	84±0.23

The result of this preliminary study was also applied to an extract of LS seed. The first attempt was on glucose oxidase (GOD) reactor. When 500 μM pure glucose solution, ultra filtrate of the extract of LS seed, and alumina-treated extract spiked with glucose, were injected sequentially in to 50 μL GOD reactor, the response of the reactor was almost the same in each case for ca. 40 injections (Fig.4.1). This indicates that there is no or very low adsorption of proteins on the reactor.

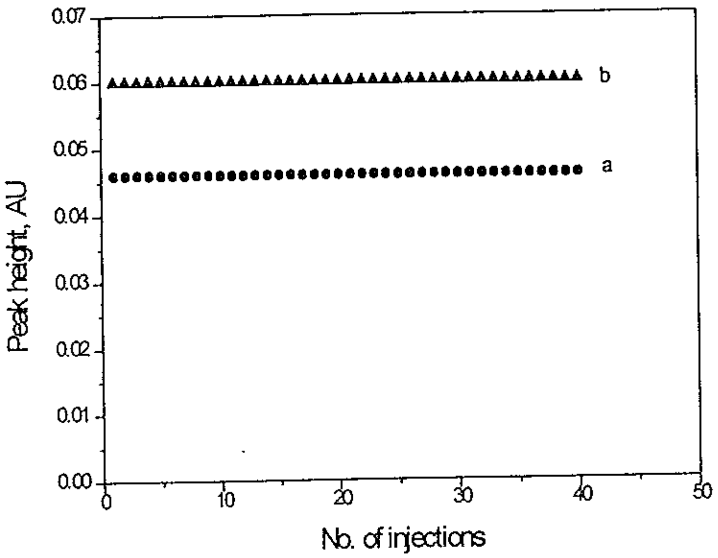


Fig. 4.1. Response of GOD (a) and GLOD (b) reactor to the off-line, alumina-treated extract of grass pea at pH 7, total flow rate 0.41 mL/min injection volume 20 µL.

The application of this result to the enzyme reactor of interest (GLOD reacor) indicated that there is no observed decay in activity of the enzyme for the specified number of injections of crude extract of LS seeds (Fig.4.1) .

Conditions for the separation of proteins on a strong anion exchanger (SAX) were chosen on the basis of the isoelectric point and stability of proteins [53]. At the isoelectric point pI the protein contains an equal number of cationic and anionic groups and sorption is minimum on hydrophillic ion-exchangers. Effective sorption appears only at a pH which is at least one unit lower (behaving as a cation) or one unit higher (behaving as anion) than the pI at low ionic strength of the solution. The pI of BSA is 4.6 and effective sorption on SAX is expected above pH of 5.6. In this work, adsorption behavior of proteins and β-ODAP on anion exchanger was studied at pH 6.5. At this pH 98 % BSA was adsorbed while 98% β-ODAP was eluted (Fig.4.2, Appendix 1,2,3).

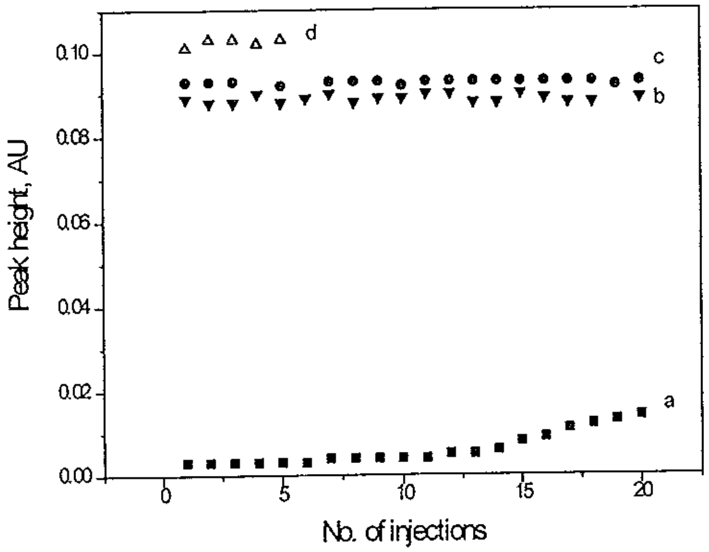


Fig. 4.2. a:BSA (0.3 mg/mL) in the presence of SAX sample clean-up column, b: β-ODAP (200 μM) in the presence of SAX sample clean-up column. c: BSA (0.3 mg/mL) in the absence of SAX sample clean-up column, d: β-ODAP (200 μM) in the absence of SAX sample clean-up column; injection volume 50 μL, carrier buffer 20 mM, pH 6.5, flow rate 0.3 mL/min; In all cases the treatment was an on-line.

As shown in Fig. 4.2 there was no significant peak height observed for the first 15 injections. This indicates that BSA was totally retained or undetectable amount might be eluted. When the number of injections was increased to 20, small peaks with gradually increasing peak height were observed. An indication of this is that the SAX column is being saturated. To study the number of injections at which the column is fully saturated, the sample was injected until an abrupt change in peak height was observed (Fig. 4.3). In the figure, it is shown that throughout fifty-nine, injections the peak height was gradually increasing. However, with further injections of the sample the peak height suddenly raised, indicating that the column was fully saturated. From the observed absorbance it was estimated that the column has a capacity to retain 98% protein within this range. Thus, in dealing with separation of proteins in a grass pea it is possible to extract a protein in such a way that 0.3 mg/mL will be within the injected sample (protein content of grass pea is ca. 30%) and 98% of it can be retained provided that the number of injections will not exceed 60 injections (injection. volume 50 μL). If an extraction is made in such a way that the crude extract contains 3 mg/mL protein, the number of injections must be decreased 10 times (6 injections) if one uses 50 μL loop. But if 20 μL loop is used, the number of injection can be increased to 15. One can extend the number of injections by eluting the adsorbed proteins. Elutionof proteins was performed within an interval of 6 and 15 injections in the former and latter case. The optimum elution solvent was 0.5 M phosphate buffer of the same pH (6.5) (Appendix 2). A large size column can also be suggested as an alternative way of extending the number of injections. This is because the more the loading of the sorbent the greater the capacity of the column to accommodate more injections.

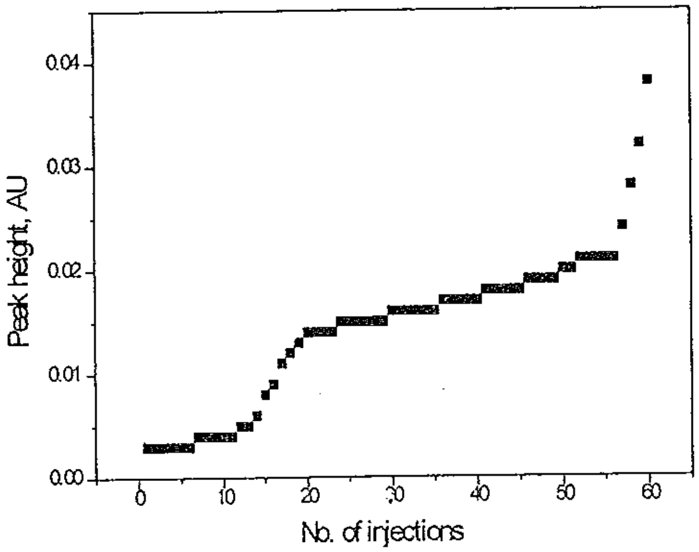


Fig. 4.3. BSA retaining capacity of SAX sample clean-up column as a function of number of injection. Concentration of BSA 0.3 mg/mL, carrier buffer 20 mM, pH 6.5, injection volume 50 μL, flow rate 0.3 mL/min. The treatment was performed on-line.

A strong cation exchanger (SCX) was also used to study the adsorption behaviour of BSA and β-ODAP as a function of pH. At pH 6.5, where BSA behaves as anion, most of it was eluted from SCX in contrast to its behaviour on SAX (Fig 4.4). As the pH was decreased to 5 (closer value to pI of BSA), ca. 40% BSA was retained indicating that cationic species became predominant. On acidification near pH 4 the adsorption behaviour changed abruptly, since in this region the cationic form was the most predominant one and, therefore strongly retained by SCX [54]. However, ca. 53% β-ODAP was also retained. As a result of this, the strong anion exchanger(SAX) was used in preference to SCX for optimum retention of proteins and elution of β-ODAP.

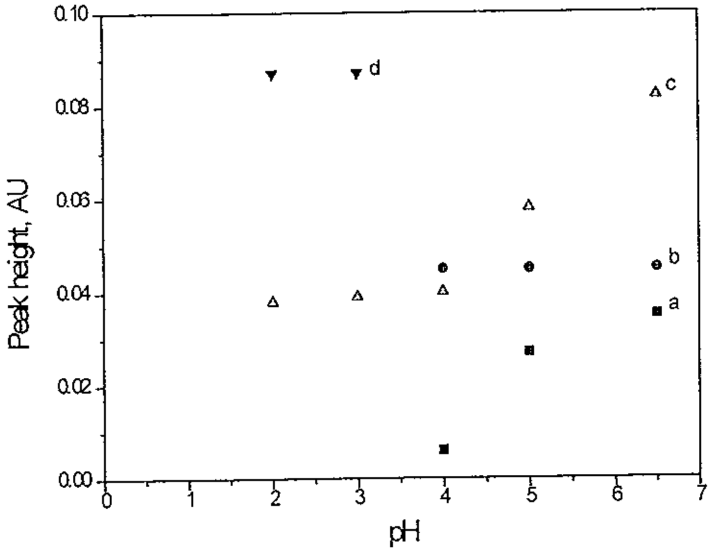


Fig. 4.4. Adsorption behaviour of BSA and β-ODAP on SCX as a function of pH. a: BSA (0.15 mg/mL) in the presence of SCX column , b: BSA (0.15 mg/mL) in the absence of SCX column, c: β-ODAP (200 μM) in the presence of SCX column, d: β-ODAP (200 μM) in the absence of SCX column. injection volume 50 μL, flow rate 0.3 mL/min, in all cases 150 μL column was used. The kind of the treatment was the same as in the previous cases (on-line).

This on-line separation using SAX was also studied by injecting crude extract of LS seed and still most proteins were retained (Fig.4.5). As it is shown in the figure, response of the FI-GLOD reactor to the extract of LS seed was almost the same for about fifteen injections when the column was used.

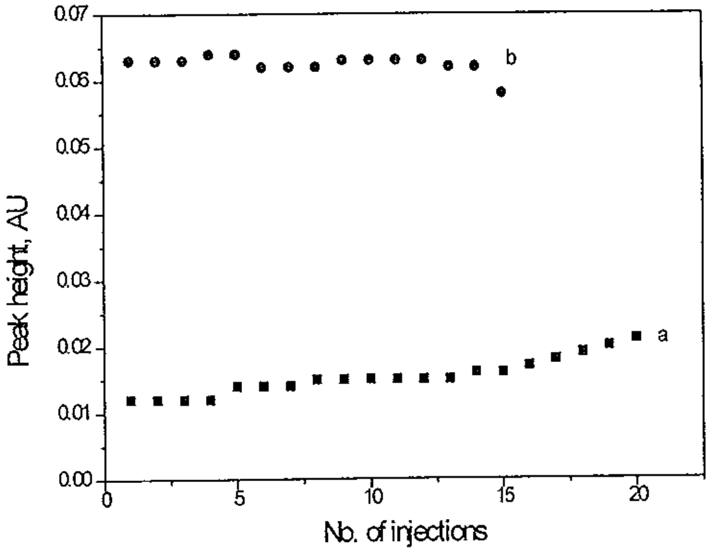


Fig. 4.5. On-line separation of proteins from extract of LS and response of FI-GLOD to β-ODAP in the presence of 150 μL SAX sample clean-up column, a: protein (3 mg/mL) in a crude extract of LS, b : β-ODAP in a crude extract; carrier buffer 20 mM, pH 6.5; injection volume 20 μL, total flow rate 0.42 mL/min.

5. CONCLUSION

When crude extract of LS seeds was injected in the presence of strong anion exchanger sample clean-up column, the response of FI-GLOD was almost the same for about fifteen injections. This indicates that almost all proteins were removed. The number of injections for which the enzyme reactor operates without being deactivated can be increased if one uses larger sample clean-up column. Off-line separation of proteins by acidic alumina could also be used although it is time consuming (at least 3 h is required) relative to shorter time required for on-line separation using SAX sample clean-up column in the FI-GLOD system.

6. REFERENCES

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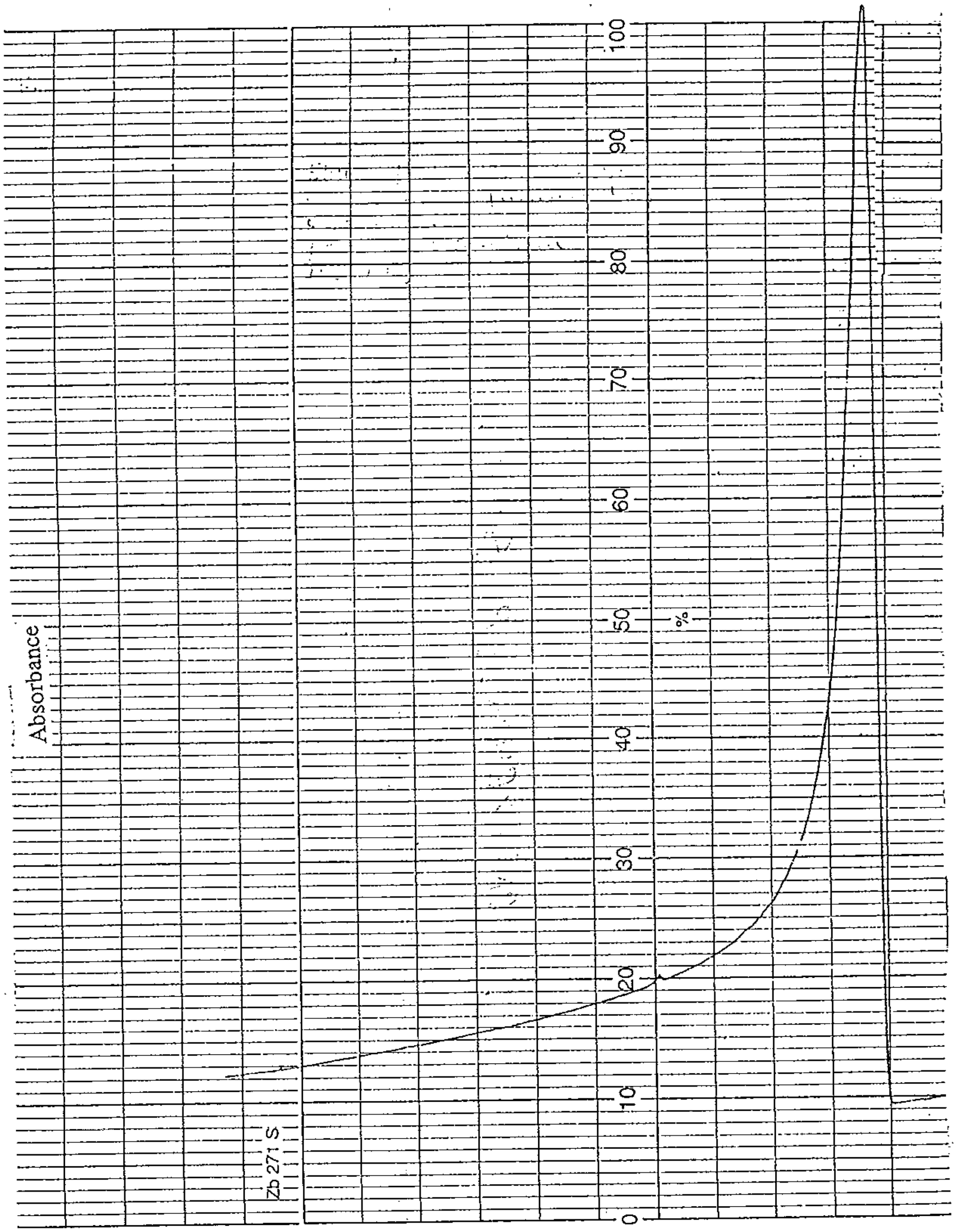
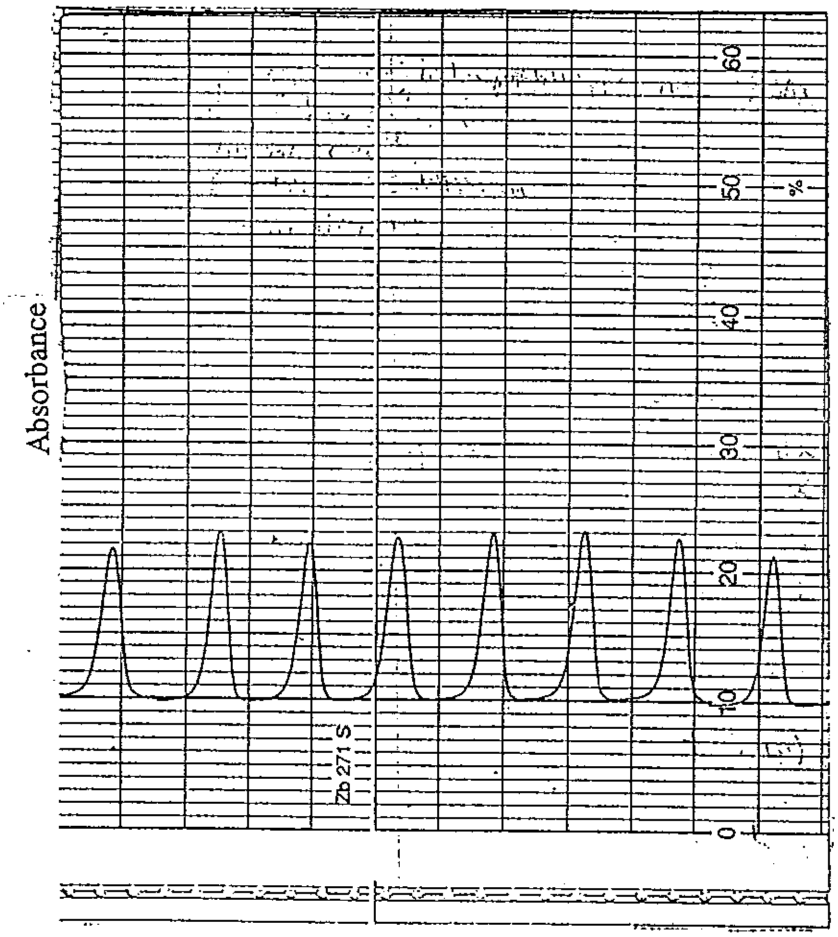
Appendix 2. Elution of the Adsorbed BSA from SAX Column

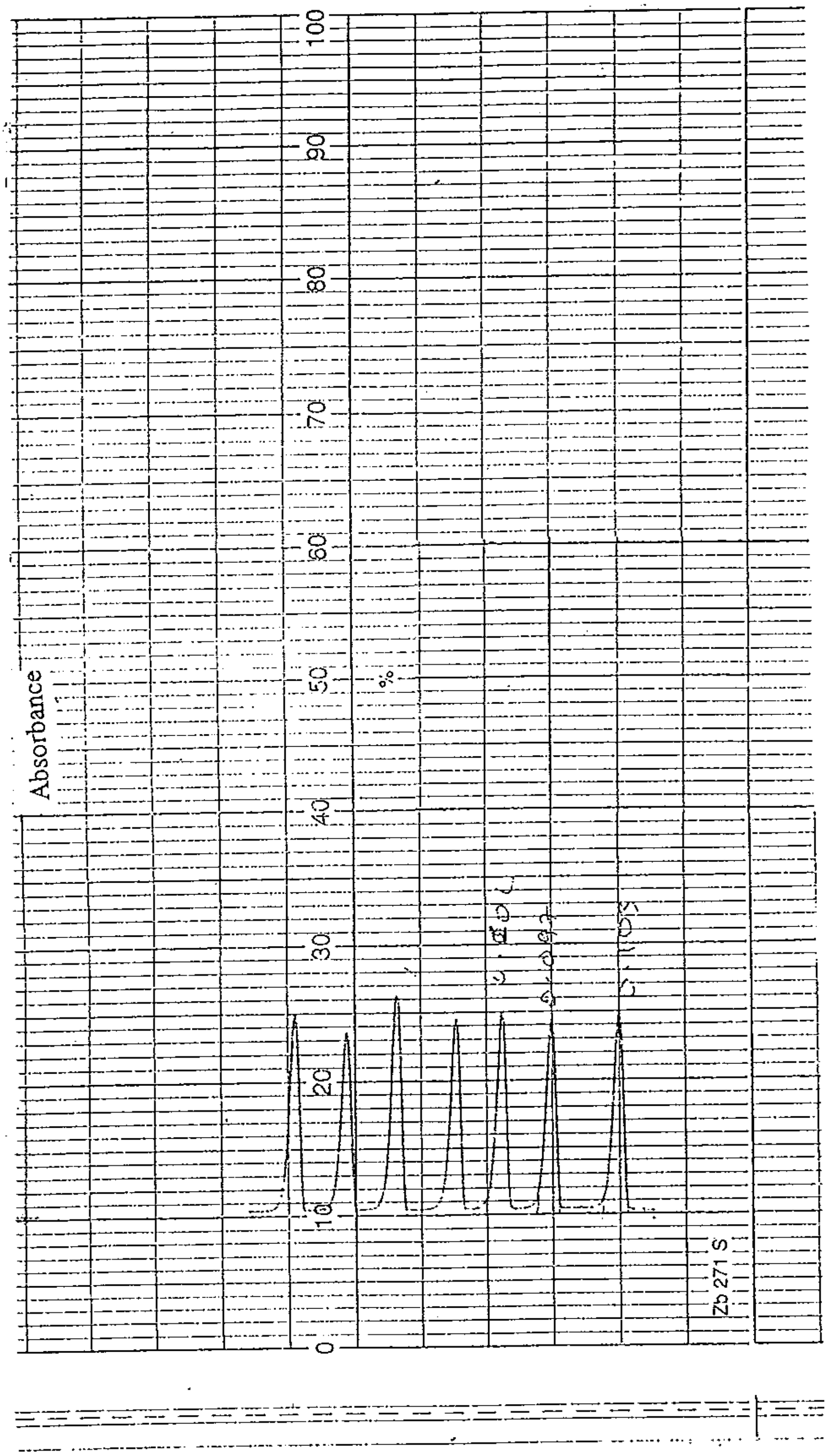


All conditions are the same as in Appendix 1. except that 0.5 M phosphate buffer was used to elute the adsorbed protein

Appendix 3. Recovery of β-ODAP after passing through SAX column.



β-ODAP(200 μM) was injected in the presence of SAX column.Other conditions are the same as in appendix 1.Scale 1 mm : 0.0035



β-ODAP (200 μM) was injected in the absence of SAX column.All conditions are the same as in the case of the presence of SAX column. Scale 1 mm : 0.0035