Bull. Chem. Soc. Ethiop. **1996**, 10(2), 129-134. ISSN 1011-3294
Printed in Ethiopia

PROTEIN PRECIPITATION METHODS FOR SAMPLE PRETREATMENT OF GRASS PEA EXTRACTS

Negussie Wodajo, Ghirma Moges* and Theodros Solomon

Department of Chemistry, Addis Ababa University,
P.O.Box 1176, Addis Ababa, Ethiopia.

(Received December 1, 1996)

ABSTRACT. Separations of protein from grass pea (*Lathyrus sativus)* extracts were made by precipitation with trichloroacetic (TCA, 1% w/v) and perchloric acids (0.46% w/v), and by an ultrafiltration technique. To test the stability of a 50 µL glucose oxidase (GOD) reactor, several injections of grass pea extracts (spiked with 300-500 µM glucose) were made into a flow injection (FI) system. The glucose-spiked extracts were detected spectrophotometrically at 512 nm, by Trinder chromogenic reaction with the product, hydrogen peroxide. No activity decay of the reactor was observed after 170 injections of the pretreated samples. On the other hand, the response of the same reactor to glucose in the crude extract showed gradual decay with no response after 36 injections due to adsorption of the grass pea protein on the reactor's surface. The study demonstrates that the stability of a reactor can be greatly improved by the pretreatments made to separate protein.

INTRODUCTION

Grass pea is a legume widely cultivated in Ethiopia and the Indian Subcontinent. It is a nutritious pulse with a protein content of 26-30%. The leguminous crop is commonly known as guaya in Ethiopia and Khesari in India. The plant is widely cultivated as a hardy crop with very little agricultural attention because it is resistant to drought and water-logging [1, 2]. *Lathyrus* foods are consumed in these regions as a cheap source of protein, particularly by the poor people. However, consumption of *Lathyrus* foods, with a major proportion in the diet for over three months causes neurolathyrism, an irreversible paralysis of human legs [3, 4]. Epidemic cases of the disease have been reported in Northwestern Ethiopia when famine had been caused by drought [4, 5]. It has been established that the cause of the disease is β-N-oxalyl-L-α,β-diaminopropionic acid, β-ODAP, found in the seeds of grass pea [6, 7].

Methods to detoxify Lathyrus foods before ingestion and the search for non- or low-ODAP varieties of grass pea have been the main important approaches to eradicate neurolathyrism [2 ,8, 9]. The most widely used method of assaying β-ODAP involves its alkaline hydrolysis to L-α,β-diaminopropionic acid (DAP) [10] which is then determined colorimetrically at 420 nm, after reaction with o-phthalaldehyde (OPT). This method lacks selectivity since the non-toxic isomer, α-ODAP, also hydrolyses to DAP. The toxin isomerizes to non-toxic isomer, α-ODAP [11] at neutral pH and elevated temperatures to a level of about 40%, e.g. during cooking [12]. Hence, there has been a need for a β-ODAP-selective assay.

A flow injection (FI) method for the determination of the neurotoxin, using immobilised glutamate oxidase reactor, was recently reported [13]. The oxidation product, hydrogen peroxide, was used for the indirect detection of the neurotoxin using Trinder reagent and a horseradish peroxidase (HRP) reactor. The results of the assay method reflect a new advance, since glutamate oxidase is found to be specific to the β-isomer and does not act on the α-isomer. However, after

a number of injections, the crude extract of grass pea produced erratic and unreliable responses which was attributed to adsorption of proteins and other macromolecules in the reactor. Moges and Johansson used ultrafiltration membranes for separating protein from the extract [13]. More recently, Belay reported that the response of the FI system decreased to 30% of the original response after 50 injections of the crude extract into a glutamate oxidase reactor [14]. But the ultrafiltration protein separation technique is not commonly available in many laboratories, particularly in the countries affected by lathyrism. Common alternative methods of separating proteins from the extract has, therefore, been necessary. Protein precipitation from grass pea extracts has been made in this study, together with the ultrafiltration method. However, reactor stability tests were made not on glutamate oxidase, but on the much more common and cheaper enzyme, glucose oxidase, GOD. We report the effect of removing protein from grass pea extracts by precipitation with trichloroacetic acid (TCA) and perchloric acid on the enhanced stability of a 50 μL GOD reactor.

EXPERIMENTAL

Reagents. 4-Amino antipyrine, AP (BDH), 2,4-dichlorophenol, DCP (Riedel-DeHaen), and D-(+)-glucose (Sigma) were used as received. 2,4--Dichlorophenol-6-sulphonate (DCPS) was synthesized from DCP and concentrated sulphuric acid according to the method of Barham and Trinder [15].

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 and 1 mg/100 mL of horseradish peroxidase, HRP (E.C. 1.11.1.7, 290 purpurogallin U/mg solid, Sigma). The carrier in the flow system was the same phosphate buffer with 0.5 mM EDTA.

The Lowry protein assay reagent [16] consisted of reagent A: 0.5 g of copper sulphate and 1 g of sodium citrate solution per 100 mL of water; reagent B: 20 g of sodium carbonate and 4 g of sodium hydroxide per 1 L of water; reagent C: 50 mL of reagent B mixed with 1 mL of reagent A; and reagent D: dilute (1 + 1) Folin-Cicalteau reagent (Sigma).

Immobilization of enzyme. 100 mg of Controlled pore glass (CPG-10 with 515 Å pore size, Serva) was silanized with 10% 3-aminopropyl-triethoxysilane (Sigma) in toluene using a reported procedure [17]. 100 mg of the silanized CPG was activated at reduced pressure with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7). 1 mg of glucose oxidase, E.C. 1.1.3.4 (GOD, from *Penicillium notatum*, 767 mmol/s per g protein, VEB LCA-Feinchmie Sebnitz) was dissolved in 3 mL of 0.1 M phosphate buffer and added to 50 mg of the glutaraldehyde-activated CPG. Packing of the immobilized enzyme was made in 50 μL plexiglass tube, i.d. 2.0 mm, for flow injection applications. To study the effect of enzyme loading on reactor stability, 10 mg of GOD was also immobilized onto 100 mg of the activated CPG and packed in a 150-μL reactor. When not in use, the reactors were stored in a pH-7 phosphate buffer at 4 °C.

Flow injection apparatus. The assembly of the flow injection system, shown in Fig. 1, consisted of a two channel peristaltic pump P (Gilson, Model M312) and Rheodyne 7125 injection valve (S, with a 20-μL sample loop) and the 150 or 50 μL GOD reactor. A mixing T-joint (M) and a coiled tube (C) were inserted between the reactor and a flow through UV-Vis spectrophotometer detector (LKB 2151). Connections in the FI system were made with Teflon tubes of 0.5 mm i.d. The flow rates were 0.6 mL/min for the carrier and 0.24 mL/min for the reagent streams.

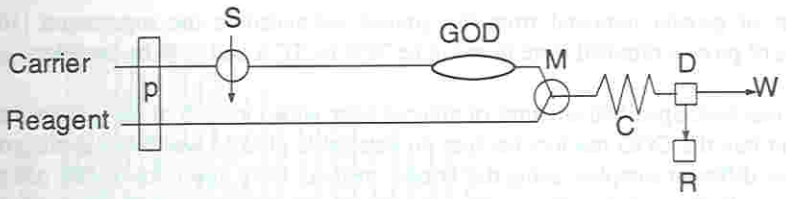

Figure 1. Flow injection manifold for studying the stability of GOD reactors upon injections of grass pea extracts.

Extraction of protein from grass pea . 40 mg of grass pea powder were taken into 10 mL of 0.1 M phosphate buffer (pH 7) and extraction was made for 2 h with stirring over ice. The mixture was filtered with a 0.45-μm membrane filter. This procedure of obtaining the crude extract was the same as the one employed for the extraction of β-ODAP from grass pea [13. 14].

Protein precipitation and residual protein assay. To 4 mL of buffer extract of grass pea kept over ice, 1 mL of 5% TCA was added and kept for thirty minutes. The mixture was centrifuged to separate the precipitate and the supernatant solution was used for assaying the residual protein. In protein precipitation by perchloric acid, 12 mL of crude extract was mixed with 1 mL of 6% perchloric acid at 4 °C.

The remaining protein in the supernatant was estimated by the Lowry method [16]. To 0.5 mL sample, 2.5 mL reagent C was added. After 10 min, 0.25 mL reagent D was added and absorbance was read at 750 nm after 30 min, using the ultrafiltrate of the extract as a blank. Ultrafiltration of the extract was made with ultrafiltration membrane (Amicon, MW cut-off value 10,000) [13].

Tests on reactor stability. To test the stability of both the 150 and 50 μL GOD reactors, aqueous β-glucose solution (300-400 μM) was added to the ultrafiltered, and the TCA- and perchloric acid-treated grass pea extracts and 130 - 140 injections were made to the FI system. As final test, 50 injections of the crude extracts, containing 300- 400 μM glucose, were Also made into the reactors.

Initially, five different crude extracts of grass pea samples were directly assayed for glucose by the Trinder method in a 3-mL cuvette. The same samples, after ultafiltration, were also injected into the two-channel FI system, with 150 μL GOD reactor, to detect any glucose.

RESULTS AND DISCUSSION

Protein separation from grass pea extracts. Trichloroacetic and perchloric acid are believed to form insoluble salts with the positively charged amino groups of the protein molecules at a pH below their isoelectric point [18]. The precipitation pH of these two acids was maintained above 2.45. This is to optimize a condition for future applications in assaying β-ODAP as a substrate to glutamate oxidase reactor, because the toxin hydrolyses at more acidic conditions [19]. When the concentrations of the precipitants, added to the extracts, were 1% for TCA and 0.46% for perchloric acid, the supernatant solutions were found to be of pH 2.6 and 2.5 respectively. This was adopted for precipitating the protein in the extract.

After centrifugation to isolate the precipitated protein, the Lowry method was used to evaluate

the amount of protein removed from the amount estimated in the supernatant [16]. The percentages of protein removal were found to be 78% by TCA and 73% by perchloric acid.

Stability of reactors. Specified amounts of glucose were added in each of the grass pea extracts for injection into the GOD reactors because no detectable glucose was found in the grass pea extracts (five different samples) using the Trinder method. Fifty injections of 400 μM glucose in water were initially made into the reactors to evaluate the FI responses. The first stability test with the extracts was made on the 150 μL GOD reactor. Fifty injections each of the grass pea extracts, after ultrafiltration, TCA- and perchloric acid-pretreatment (with 300-500 μM glucose) were made into the reactor. The FI responses of equimolar glucose in water and in *Lathyrus* extracts were found comparable. No detectable decay of FI response was observed after all the injections. Fifty injections of the crude extract (400 μM glucose) were further made into the same reactor and no decrease in the FI response was again observed. Hence, the FI response after the enzyme reaction was unaffected after a total of 190 injections (see Fig. 2). Injection of the crude extract did not exhibit any decrease in FI response because any activity decay remained unnoticed due to high enzyme loading in the reactor. The fact that the response of the FI system with the 150 μL GOD reactor is unaffected by the 50 injections of the crude grass pea extract reflects that the stability of an enzyme reactor can be enhanced by high enzyme loading.

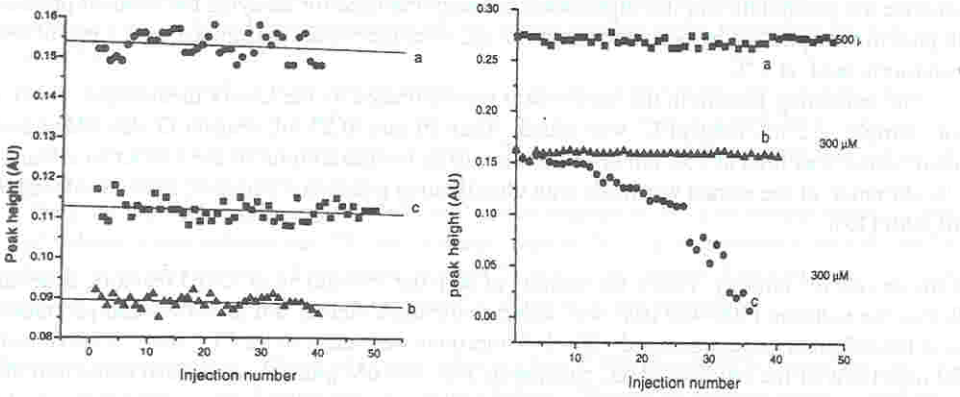

Figure 2. Plots of flow injection peak heights for added glucose, with the 150 μl GOD reactor, against injection number: (a) pure glucose solution (500 μM and b) perchloric acid treated extract (300 μM glucose) and (c) crude extract (400 μM glucose).

Figure 3. A plot of peak heights for added glucose, with the 50 μl GOD reactor, versus injection number of grass pea extracts for (a) perchloric acid-treated (500 μM glucose), (b) TCA-treated (300 μM glucose), and (c) crude extract (300 μM glucose).

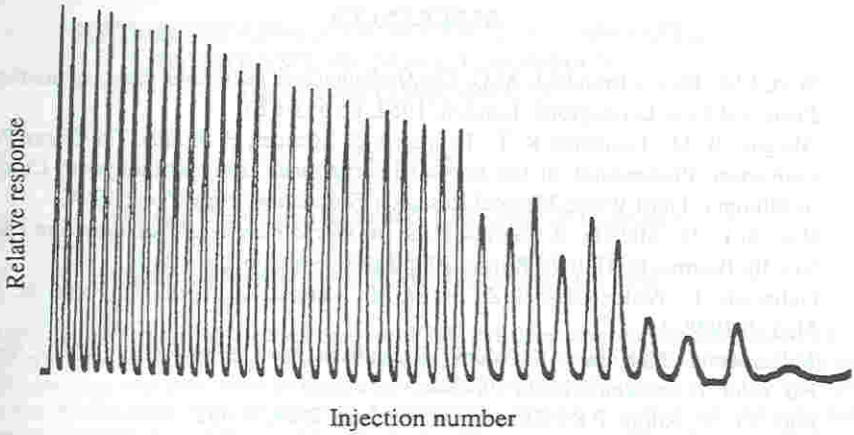

Figure 4. Decaying flow injection records with the 50-μL GOD reactor for a crude grass pea extract containng 300 μM glucose.

It was, therefore, necessary to employ a reactor with a small enzyme loading to observe the effect of the crude extract. Thus, the size of the GOD reactor was reduced to 50 μL, and during immobilization onto 50 mg of CPG, only 1 mg (46 U) of GOD was used. This was about the same activity of glutamate oxidase (42 U) used by Moges and Johansson for immobilization onto CPG [13]. The same FI runs, 40-50 each, were made with the small GOD reactor for the ultrafiltered, and TCA- and perchloric acid pretreated grass pea extracts. Essentially unchanged FI responses were recorded for a given sample (300 - 400 μM glucose), see plot a and b in Fig. 3. However, injections of the crude extract showed a gradual decrease in response to the added glucose, and no response was observed after the thirty six injection (see Fig. 3c). Recorder tracings for the FI peaks of the crude extract are also shown in Fig. 4.

CONCLUSION

The results of this investigation illustrate that the stability of an enzyme reactor can be improved by separating the protein from Lathyrus extracts, with TCA and perchloric acid precipitations. High enzyme loading also prolongs the stability of a reactor. The results of the study paves the way to alternative approaches, other than ultrafiltration, towards the application of the target reactor, glutamate oxidase, when a flow injection system is to be employed for assaying β-ODAP.

ACKNOWLEDGEMENTS

G.M. acknowledges Dr Kusakabe of Yamasa Corp., Japan for the donation of glutamate oxidase. This project was supported by the Swedish International Development Agency (SIDA). The Ministry of Health sponsored the scholarship to N.W.

REFERENCES

1. Watt, J.M.; Breyer-Brandeijk, M.G. *The Medicinal and Poisonous Plants of Southern and Eastern Africa*, Livinsgtone: London; 1962, pp 613-620.
2. Abegaz, B. M.; Haimanot R. T.; Palmer, V.S, Spencer, P. S. (ed), *The Grass Pea and Lathyrism*, Proceedings of the Second International *Lathyrus*/Lathyrism Conference in Ethiopia, Third World Medical Research Foundation: New York; 1994.
3. Rao, S. L. N.; Malathi, K.; Sarma, P. S. in *World Review of Nutrition and Dietetics*, Vol 10, Bourne, G. H. (ed), Karger AG: Basel, 1969, pp 214 - 238.
4. Gebre-ab, T.; Wolde Gabriel, Z.; Maffi, M.; Ahmed, Z.; Ayele, T.; Fanta, H. *Ethiop. Med. J.* **1978,** 16, 1.
5. T. Haimanot, R.; Kidane, Y.; Wuhib, E.; Kalissa, A.; Alemu, T.; Zein, Z. A.; Spencer, P.S. *Inter. J. Epidemol.* **1990,** 19, 664.
6. Rao, S.L.N.; Adiga, P.R.; Sarma, P.S. *Biochem.* **1964**, 3, 432.
7. Murti, V. V. S.; Seshadri, T.R.; Venkitasubramanian, T. A. *Phytochemistry* **1964,** 3, 73.
8. Rosenthal, G., Lambien, F. (eds), Abstracts, *International Workshop on Ecology and Biochemistry of Non-protein Amino acids from Plants*, University of Ghent: Belgium.
9. Lambein, F.; Yusuf, H.K.M. (eds) *Lathyrus and Lathyrism, Progress and Prospects*, 2nd International Colloquium on Lathyrism in Bangladesh, University of Dhaka: Dhaka, 1995.
10. Rao, S.L.N. *Anal. Biochem.* **1978**, 14, 387.
11. Chase, R.A., Pearson, S., Nunn , P.B., Lanton, P.L. *Neurosci. Lett.* **1985,** 55, 89.
12. Khan, J.K., Kebede, N., Lambein, Y.H., De Bruyn, A. *Anal. Biochem.* **1993,** 208, 237.
13. Moges, G., Johansson, G. *Anal. Chem.* **66** (1994) 3834.
14. Belay, A, MSc Thesis, Addis Ababa University: Addis Ababa (Ethiopia), 1995.
15. Barham, D.; Trinder, P. *Analyst* **1972,** 96, 142.
16. Scopes, R.K.; *Protein Purification: Principles and Practice*, 3rd ed., Springer-Verlag: New York; 1994.
17 Wettal, H.H. *Anal. Chem.* **1974**, 46, 602A.
18. Blanchard, J. *J. Chromatogr.* **1981**, 226, 455.
19. Abegaz, B. M.; Nunn, P. B.; De Bruyn, A.; Lambein, F., *Phytochemistry* **1993**, 33 1121.