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SHORT COMMUNICATIONS

COMPARATIVE STUDY OF AQUEOUS EXTRACTIONS OF THE NEUROTOXIN β-ODAP FROM GRASS PEA ASSAYED BY FLOW INJECTION ANALYSIS

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ABSTRACT. Three different aqueous extraction procedures were investigated for the determination of the neurotoxin β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) from grass pea (*Lathyrus sativus*). Samples of the same grass pea powder were extracted at room temperature with water, phosphate buffer, and with water using a sonicator at 55 °C. β-ODAP was determined by flow injection (FI) after its selective oxidation in an immobilized glutamate oxidase (GlOD) reactor (any glutamate in the sample would be destroyed in a small GlOD-catalase prereactor). The oxidation product, hydrogen peroxide, was determined colorimetrically at 512 nm through a chromogenic reaction. The three extraction methods for the same sample yielded similar peak absorbances due to the detected β-ODAP in the FI-GlOD system. Extraction in phosphate, with or without EDTA, yielded the same toxin content in grass pea. Isomerization of β-ODAP (80° C, 1 h) to the non-toxic α -ODAP, in the *Lathyrus* extracts in phosphate buffer, yielded a typical β : α equilibrium concentration ratio (61% : 39%).

INTRODUCTION

Grass pea is a legume widely cultivated in Ethiopia, India and its neighbours. The edible pulse provides high quality protein up to about 30%. β-ODAP, the neurotoxin which causes a disease called neurolathyrism is found in grass pea [1]. Irreversible paralysis of the legs in humans (neurolathyrism) occurs when the *Lathyrus* food is the major part in the human diet [2]. The disease was reported to be in epidemic proportions in Northwestern Ethiopia, when famine, caused by drought, prevailed in the region [3].

Rapid and selective assay for the toxin is sought for by *Lathyrus* researchers to minimize or eliminate the content of β-ODAP in *Lathyrus*-based foods. Rao's colorimetric method is the most common method and involves an extraction procedure that originally employed 60% alcohol [4]. Different extraction procedures of the neurotoxin have been used to modify the Rao colorimetric procedure [5], including water extraction with sonication at 55 ° [6].

Recently, a rapid flow injection method for assaying the neurotoxin has been reported using immobilized glutamate oxidase reactor [7]. Glutamate oxidase was found to selectively catalyse the oxidation of β-ODAP, without effect on its nontoxic isomer, α-ODAP [7, 8]. The FI-enzymatic method is significant achievement because the Rao method makes no difference between the two isomers. Quantitative extraction of the neurotoxin in phosphate buffer (pH 7) was reported in the flow injection enzymatic method [7]. Kinetic and equilibrium data on the off-line isomerization of the toxin was reported using the β-ODAP selective GlOD reactor in a flow system [9]. In this short report, we present a comparison of the results of aqueous extractions of the neurotoxin from powdered grass pea samples for the enzymatic flow injection method of assaying β-ODAP.

EXPERIMENTAL

Reagents. 4-aminoantipyrine (AP) (BDH) was used as received. 2,4-dichlorphenol-6-sulphonate (DCPS) was synthesized from 2,4-dichlorophenol, DCP (Riedel-DeHaen) and concentrated sulphuric acid, according to Barham and Trinder [10]. β-ODAP.HCl was synthesized according to Rao [11]. The reagent for the FI system was prepared in 0.1 M phosphate buffer (pH 7), as described before [5, 9] and consisted of 2.5 mM DCPS, 0.5 mM 4-AP, 0.5 mM EDTA and 0.01 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).

Immobilization of enzymes. The reactors for the FI system were prepared as follows. Controlled pore glass (CPG-10, pore size 515 Å, Serva) was added to 10% 3-aminopropyl(triethoxy)silane (Sigma) in sodium-dried toluene and refluxed for 40 min over a boiling water bath [12]. Silanized CPG, 180 mg, was activated with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7) at reduced pressure for 30 min. Seven mg of glutamate oxidase (GlOD, *Streptomyces sp.*, E.C. 1.4.3.11, 6.8 units/mg; from Yamasa Corp., Japan) was dissolved in 3 mL of 0.1 M phosphate buffer at pH 7. The enzyme solution was added to 180 mg of the glutaraldehyde-activated CPG [7, 9]. About 120 mg of the immobilized glutamate oxidase was packed in a 250 μL plexiglass tube (i.d. 2.0 mm) for flow injection applications. Storage of the reactor was made in 0.1 M phosphate buffer at pH 7 and 4°. One mg of catalase (from Bovine liver, E.C. 1.11.1.6, 19,900 units/mg solid (Sigma), dissolved in 3 mL 0.1 M phosphate buffer, was immobilized on 50 mg of CPG using the same method used for GlOD. Immobilized GlOD and Catalase (25 μL each) were mixed and packed in a 50-μL prereactor.

Flow injection set-up. The flow injection assembly for assaying β-ODAP has been reported earlier [7]. Along the carrier stream were a Rheodyne 7125 injection valve (sample loop 20 μL), 50-μL GlOD/catalase prereactor and a 250-μL GlOD reactor. The carrier and the reagent streams were delivered by two-channel peristaltic pump (Gilson, Model M312) to meet at a confluence point in a mixing tee. A coiled tube was inserted after the mixing tee. Detection of the product was made at 512 nm in a flow-through UV-Vis spectrophotometer (LKB 2151). All connections in the FI system were made with Teflon tubes (0.5 mm i.d.). The flow rates were 0.3 mL/min for the carrier and 0.12 mL/min for the reagent stream.

Extraction of β-ODAP from grass pea. β-ODAP was extracted from 40 mg of grass pea powder in 10 mL of 0.1 M phosphate buffer (pH 7) with and without 500 μM EDTA. The extraction was made over an ice bath by agitation with a magnetic stirrer for 2 h. The same procedure was followed for extraction of the neurotoxin in 10 mL of double distilled water. Particulate matter was removed by filtration through a 0.45-μm membrane filter. Extraction of the toxin from 80 mg of powder in water was also made for 2 h, using a sonicator at 55° [6]. Whatman No. 542 filter paper was used to remove particulate matter. Ultrafiltration membranes (Amicon, MW cut-off value 10,000) were used to remove protein and other macromolecules from all the extracts using a centrifuge at 4000 rpm [7]. Prior to injection, the ultrafiltrate from the sonicated extract was diluted in such a way that it would contain the same amount of grass pea sample as in the other extracts.

Isomerization of β-ODAP in grass pea extracts. 500 μL of each of the ultrafiltrates of buffer extracts were placed in five different Eppendorf tubes and heated in a water bath at 80° for one hour. The samples were cooled to room temperature. The unisomerized β-ODAP was determined

(n = 2) by injecting the sample at room temperature into the flow system. As a control experiment, the same thermal experiment was made for 300 μM glutamate and injected into the flow injection without GlOD-catalase prereactor.

RESULTS AND DISCUSSION

Comparison of extraction efficiency. Three different procedures were investigated to examine the level of extraction of β-ODAP. Five grass pea seed samples were extracted in water, in phosphate buffer (25°), and in water using a sonicator at 55°. The absorbances of the injected samples are shown in Table 1.The results reflect a maximum difference of 5% from the lowest absorbance

Table 1. Comparison of the response of GlOD reactor towards β-ODAP extracted using three different extraction conditions.

Call No*	Peak height (Absorbance)		
	Buffer extract	Water extract	extraction with sonication
71	0.062	0.060	0.059
72	0.058	0.061	0.058
74	0.055	0.057	0.056
75	0.047	0.046	0.048
76	0.048	0.052	0.050

*Sample code GP GPE(Adet) G/94

value for given sample. Hence, simple extraction in distilled water is as effective as phosphate extraction and water extraction with sonication. Higher temperature extraction could cause partial isomerization of β-ODAP but the results of the flow injection peaks are not generally lower than those at 25°.

Table 2. Determination of β-ODAP extracted in phosphate buffer with and without EDTA (n = 2).

Sample code (Acc/ser.)	% of β-ODAP found by the FI system	
	Buffer	Buffer + EDTA
385/504	0.696	0.694
392/505	0.672	0.696
462/527	0.684	0.681

EDTA is normally added to reagent streams to bind metal ions so that enzyme reactor(s) would not be inactivated by adsorbed metal ions. In the present study three grass pea seed samples were extracted in the same buffer with and without 500 μM EDTA. The percentage of β-ODAP in each case is presented in Table 2. The results of this observation shows that the presence of EDTA does not have any special advantage during the extraction. It may not, therefore, be necessary to use a buffer containing EDTA for the extraction of β-ODAP in grass pea.

Thermal isomerization of β-ODAP in grass pea. Table 3 shows the percentages of unisomerized β-ODAP for four samples heated in a water bath at 80° (1 h).

These results show that the equilibrium concentration of the toxin after heating lies in the range 57.8-62.8%, mean 61%. Hence, the data for the grass pea extracts in Table 3 compares well with earlier reported data on the isomerization of β-ODAP in phosphate buffer at pH 7 (59-60% β-ODAP), determined by FI-GlOD reactor system [7, 9].

Table 3. Percent isomerization of β-ODAP after heating samples at 80 ° for 1 h (n = 2).

Call. No*	% β-ODAP unisomerized
71	57.8
72	62.4
74	61.8
75	62.8

*Sample code GP GPE(Adet) G/94.

Injection of the control, glutamate solution, was made after removing the GlOD-catalase prereactor. The response to 300 μM glutamate was the same before and after heating, confirming that no change occurred in the solution.

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REFERENCES

1. Rao, S.L.N.; Malathi K.; Sarma, P.S. In *World Review of Nutrition and Dietetics,* Vol. 10, 214-238 (Ed. G.H. Bourne) Karger AG: Basel; 1969.
2. Rao, S.L.N.; Adiga, P.R.; Sarma, P.S. *Biochem.* **1964**, 3, 432.
3. Haimanot, R.T.; Kidane Y.; Wuhib E; Kassina, A.; Alemu, T.; Zein, Z.A.; Spencer, P.S. *J. Epidemiol.*, **1990**, 19, 664.
4. Rao, S.L.N. *Anal. Biochem.* **1978**, 86, 386.
5. Hussain, M.; Chowdhurry, B.; Haque, R; Wouters G; Campbell C.G. *Phytochem. Anal.* **1994**, 5,247.
6. Abegaz, B.M.; Alemayehu, G.; Yigzaw, Y. in *Lathyrus and Lathyrus and Lathyrism , A Decade of Progress,* R. Tekle Haimanot and F. Lambein (Eds) University of Ghent, Ghent, 1997, p 75.
7. Moges, G.; Johansson, G. *Anal. Chem.* **1994**, 66, 3834.
8. Moges, G.; Johansson, G.; Solomon, T. *Anal. Lett.* **1994**, 27, 2207.
9. Belay, A.; Moges, G.; Solomon T.; Johansson ,G. *Phytochem.* **1997**, 45, 219.
10. Barham, D.; Trinder, P. *Analyst* **1972**, 97, 142-145..
11. Rao, S.L.N. *Biochem.* **1975**, 14, 5218.
12. Emnèus, J. *Ph.D. Thesis*, University of Lund (Sweden): 1992.