A Rapid Putrescine/Cadaverine Test for Decomposition in Fisheries Products

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FINAL REPORT
Mississippi/Alabama Sea Grant

Robert C. Bateman, Jr. and Margot Hall
"A Rapid Putrescine/Cadaverine Test for Decomposition in Fisheries Products"
2/1/97-1/31/98 (one year grant extended to 7/31/98)
NOAA#NA56RG0129

Project Goal and Objectives (from original proposal)

The overall goal of this project is to produce a sensitive, accurate, rapid and convenient test for putrescine and cadaverine levels in fresh seafood and seafood products. This goal could be met by producing either a separate test for each amine or a combined test which reacts with both amines.

i) *Isolation of amine oxidases and determination of the optimal oxidases for putrescine & cadaverine:*

The test(s) will be based on the enzymatic breakdown of putrescine and cadaverine by the appropriate amine oxidases. Amine oxidases from several sources will be isolated by literature methods and tested to determine which one will work best with the proposed test(s). Large scale production of the optimal enzyme will be accomplished at this time.

ii) *Development of a chemiluminescent technique to quantitate diamine oxidation:*

The amine oxidase reaction with putrescine and cadaverine will be coupled to the luminol/potassium ferricyanide reaction to produce a burst of light when the diamines are present. Conditions such as pH, stabilizers, concentrations of reagents, timing of the reactions steps, storage conditions, etc will be worked out at this stage.

iii) *Performance characterization of the test(s):*

The tests(s) developed above will be assessed for sensitivity, precision, accuracy, and linearity using standard solutions of putrescine and cadaverine, and tuna spiked with known concentrations of putrescine and cadaverine. The tests will be tested for interference from other amines, such as histamine, spermine, and spermidine, and for stability on storage.

iv) *Testing with spoiled tuna:*

The test(s) developed above will be assessed with fresh tuna and tuna following controlled decomposition. Results will be compared with those obtained using a reference GLC method.

Abstract:

A chemiluminescent assay for diamines was developed using plant diamine oxidase coupled to the horseradish peroxidase/luminol system. The reaction conditions were optimized for pH, type of buffer, injection volumes, incubation time, and integration time. Use of pea seedling diamine oxidase allowed the determination of low levels (< 1 ppm) of diamines in standards, but measurement of diamines spiked into tuna extracts was confounded by interfering components in the extract. Attempts to eliminate or circumvent these components have thus far proven unsuccessful.

Materials and Methods:

Light intensity was measured using a TD-20/20 Luminometer manufactured by Turner Designs. The output data was sent to an attached printer. The initial parameters are listed in table 1 and they will remain the same unless specified otherwise.

Integration Time	Delay Time	Number of replications	Mode	Sensitivity	Injection Volume
60sec	0sec	1	STD	40.9%	200µL

Table 1: Initial Settings

The reagents obtained from Sigma Chemical Company included luminol (5-amino-2,3-dihydro-1,4-phthalazinedione or $C_8H_6N_3O_2Na$), Horseradish Peroxidase(HRP)—Type X, and 30.9% Hydrogen Peroxide with lot numbers 71H37861, 55H9584, and 27H3473, respectively. Stable Peroxide LBA was purchased from Pierce Chemical Company (lot # 96112662).

Diamine oxidase from 14 day pea seedling cotelydons was partially purified by ammonium sulfate precipitation (30-60%) and phenyl sepharose chromatography using a 30-0% ammonium sulfate gradient. Diamine oxidase from soybean was partially purified from mature soybean plants by 45-70% ammonium sulfate precipitation. During purification, diamine oxidase activity was measured using a putrescine/HRP/ABTS assay protocol. Fresh albacore and yellowfin tuna were obtained from a local seafood shop and stored at -20°C until use.

The luminol cocktail was made fresh daily by combining a stock solution of luminol (also fresh), buffer, and HRP. The concentration, volumes, and pH of all reagents are specified in each experiment while the amount of HRP used will be constant at 5 units per 1000 µL of cocktail unless otherwise noted.

Standard Chemiluminescent Assay Procedure: The diamine sample (45uL) was mixed with 5uL of the diamine oxidase enzyme. This was incubated for a given amount of time at ambient temperature, normally 1 minute, to allow oxidation of the diamine. The test tube was then placed in the luminometer and the lid was closed. The "Go" button on the luminometer keypad was pressed and

200μL of luminol cocktail was automatically injected into the test tube. Upon mixing, a burst of light was produced and the intensity of the burst was recorded by the luminometer, normally with an integration time of 4 seconds.

Results:

Optimization of detection system (HRP/luminol) using hydrogen peroxide:

The integration time was the first parameter to be optimized. The final concentrations of luminol and buffer were 1 mM and 67 mM, pH 8.6. The sensitivity, injection volume, and delay time remained the same as listed above. The variation in integration time with intensity demonstrated that shorter integration time yielded higher light intensity. This was expected since the reaction is a rapid "flash" reaction. In the following experiments, an integration time of 60 seconds will be used since a strong signal is obtained during that time interval.

A variation in pH was the next condition examined. The buffer used in this run was 0.1M sodium borate buffer at pH 8 and 8.6. The higher pH, with peroxide as the substrate, gave a greater intensity signal in the 0-3000 ppm range. Luminol has previously been reported to prefer higher pH; however HRP does not work optimally at basic pH. In this case, a pH of 8.6 seemed to work better than 8.0.

The sample volume to injection volume ratio was adjusted to determine which gave the optimal light output. The volumes were changed from 200 μL peroxide:200 μL cocktail to 50 μL peroxide:250μL of cocktail yielding a 1:5 ratio. The change in volume ratio from 1:1 to 1:5 allowed for better mixing and produced a more linear response. There was a lower production of intensity, but this was not a concern since the volume of peroxide was cut fourfold.

During this next experiment, the data was transferred to a computer instead of a printer. The luminometer was configured to record five intensity measurements per second and output the data points to a text file. The data points were plotted on the graph below. A 1mM concentration of luminol in 50 mM glycine buffer at pH 8.6 was used with 5 μM and 10 μM of 30.9% peroxide. The injection volume ratio was 50 μL of 5 μM and 10 μM peroxide solution to 250μL luminol cocktail. The non-zero starting time was the lag time in data transfer between the computer and the luminometer. Since the reaction was essentially over at 5seconds the integration time was reduced from 60 seconds to 10 seconds. This graph also shows the doubling of light intensity with the doubling of peroxide concentration.

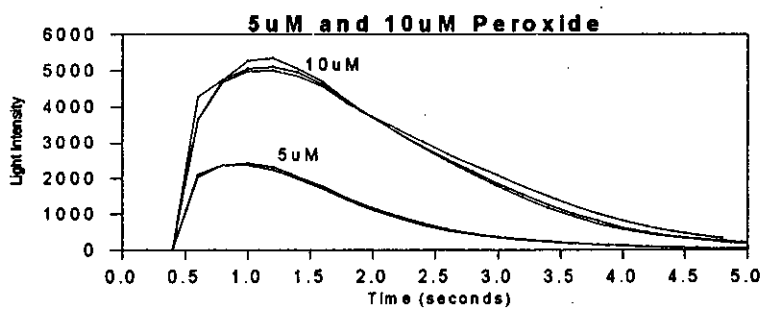


Figure 1: 5μM and 10μM peroxide reacting with the luminol cocktail.

In the next run, tris-acetic acid, sodium borate, phosphate, and glycine buffers were examined to determine which is the most appropriate. The buffers had a concentration of 0.1 M and a pH of 8.3. The light intensity was measured for varying concentrations of peroxide from 0-3000 ppm. Each buffer gave different levels of intensity, even though the reagent concentrations and volumes were the same for each measurement. The tris-acetate and glycine buffers gave the highest light output, while phosphate buffer gave the weakest.

The optimal conditions consisted of a 66uM stock solution of luminol, a 5.25unit/mL solution of HRP, and a 50mM glycine buffer at pH 8.6. These components were combined to make a cocktail in a 2:2:5 ratio, respectively. The injection volume ratio was 50 μL peroxide:200μL cocktail, with an integration time of 5 seconds. The data was collected and plotted in figure 2 below which shows a linear relation between the light output and the concentration of peroxide.

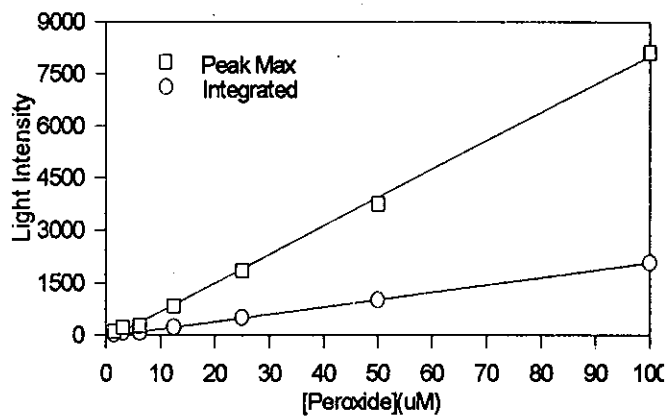


Figure 2: Light intensity versus concentration of peroxide in uM.

Optimization of Method for Diamine Detection:

Before the intensity and concentration relationship for histamine was examined, the time of incubation for histamine in pea cotyledon diamine oxidase (pDAO) was studied. It turned out that histamine needed to be incubated for 10 minutes to obtain maximum intensity whereas cadaverine and putrescine needed only 1 minute preincubation with enzyme. Using the same concentrations of luminol, HRP, and buffer optimized above the light intensity plus pDAO histamine was found to be linear in the 0-100uM range. Since the conditions determined thus far result in a linear relation between the light intensity produced and the amount of histamine present, a different diamine oxidase was then examined. Under the same conditions soybean DAO yielded the following relative numbers.

25uM diamine	**Run 1 @ 60sec (intensity)**	**Run 2 @ 60sec (intensity)**
Cadaverine	1468	1521
Putrescine	1278	1326
Histamine	29	30

This experiment confirms that the soybean DAO is more specific for cadaverine and putrescine and

can also be easily incorporated into the assay.

In the next experiment a matrix was used to ensure that the concentrations of luminol and HRP were optimal once DAO was incorporated into the assay. The experiment was performed with 1uM cadaverine. In the graph below the pea DAO results are shown. The soybean results followed the same trends. The ideal final concentration of HRP was again set at 2.5 units/mL while luminol was set at 45 uM.

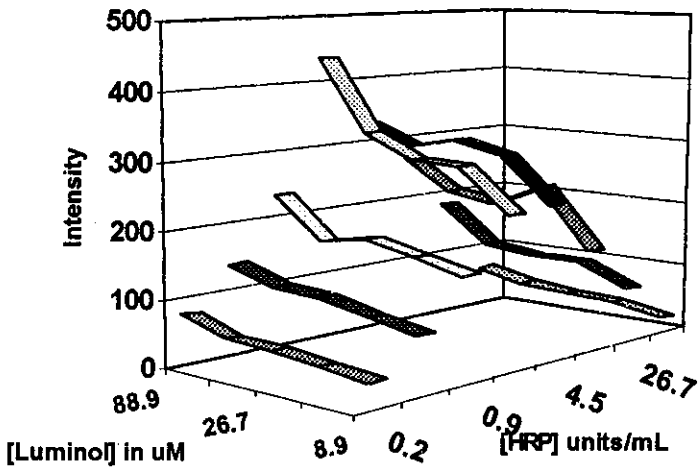
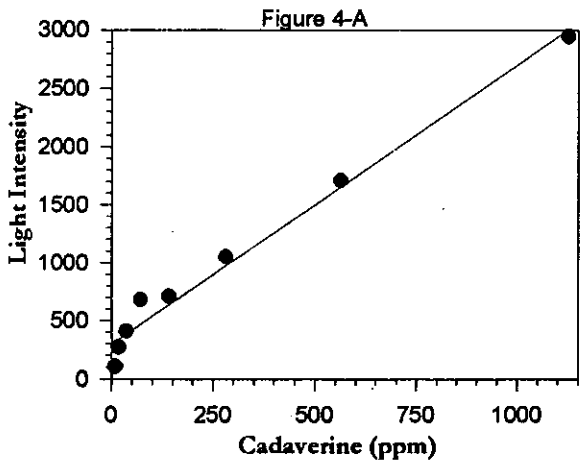
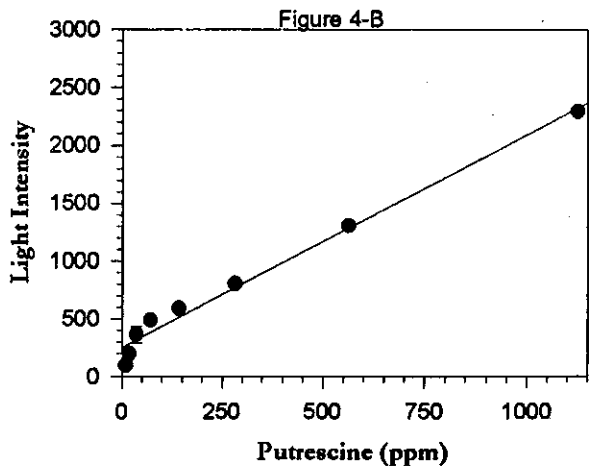
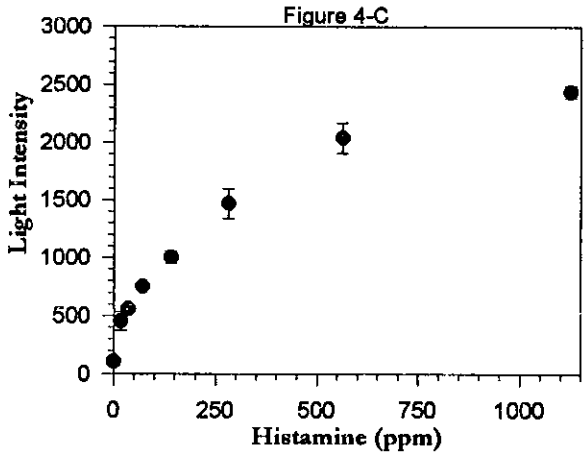


Figure 3: 3-D graph of intensity <vs> [luminol] and [HRP].

These optimal conditions were used to determine the relationship between the light intensity and the amount of histamine, cadaverine, and putrescine present in standards. The cadaverine and putrescine curves showed a linear relationship while histamine did not (figure 4A-C). This was probably due to the slower reaction of histamine. The tailing off at lower amounts was most likely due to the sensitivity limits of the instrument and/or the result of using a coupled assay system.







Detection of Diamines in Spiked Tuna Extracts

Since the object of this grant was to measure diamines in fish samples, we began by assaying diamines spiked into buffer extracts of fresh tuna. The results of these were clearly different from the same concentration of diamines in buffered standards, indicating components in the tuna extract. that interfered with the chemiluminescent assay. Attempts to eliminate the interfering components by simple dilution of the extract led to results such as those below:

Dilution of Tuna	Tuna only	Tuna w/ 10uM cadaverine
Full strength	12	129
½	175	605
¼	533	1172
1/8	863	1891
1/16	714	2168
1/32	503	1885

The trends in the data suggest that an inhibitor is present along with the unknown interfering agent. The inhibitor seemed to be diluted out quicker than the interfering agent, but the interfering agent was removed by further dilutions.

Since dilution did not seem to work, we examined cation exchange chromatography as a rapid cleanup method we have published previously for a copper chelation histamine assay. Fresh tuna homogenized in 0.025 M phosphate buffer at pH 6.5 was chromatographed on a CM cation exchange cartridge column (Waters Sep-Pak). The tuna extract was spiked with cadaverine (final concentration of 10uM) and then loaded onto the column. As a control an identical column run was performed without tuna (buffer only). The cadaverine was eluted with the 50 mM glycine buffer at

pH 8.6. These were compared with a 10uM solution of cadaverine in glycine buffer not loaded onto the column. The following data was collected:

Assay conditions	Tuna spiked pre-column	Buffer spiked pre-column	10 uM cadaverine no column
Standard	506.5	68.06	1298
	652.3	59.96	1317
DAO omitted	206.9	41.27	54.36
	232.7	73.65	72.41

This data shows that the 50mM glycine buffer is not eluting cadaverine from the column. A further attempt to elute with 0.2M glycine was also unsuccessful.

Glycine, phosphate, and borate buffers at different concentrations were used to determine the effects on the chemiluminescent assay system before they were tried as column elutants. Ten micromolar cadaverine solutions were made in each buffer without columns and assayed (run 1 and run 2). The following data was obtained:

Buffer	Run 1	Run 2	Buffer Blank	Buffer w/ DAO Blank
0.1M Borate	1264	1258	44	40
0.2M Borate	1099	1072	32	32
0.1M Phosphate	1506	1484	43	30
0.2M Phosphate	1483	1491	151	71
0.05M Glycine	1168	1106	65	56
0.2M Glycine	1130	1116	167	94

Since the concentration of buffer has little or no effect on the system, any of these tested would be suitable for eluting the cadaverine. The 0.2M borate and phosphate buffers were then used to elute the cadaverine. The borate buffer did not elute the cadaverine while the phosphate buffer readily eluted the cadaverine.

Two columns were loaded (one with tuna extract only and one with tuna extract spiked with cadaverine). The columns were washed twice with the 0.025M phosphate buffer at pH 6.5 and then eluted with 0.2M phosphate at pH 8.6. The results are displayed in the following table.

	Spiked tuna column	Tuna Column
Elutant	>9999	2366
Load	0.031	0.089
Wash 1	58.72	4.700
Wash 2	85.59	57.61
Elutant w/ 0.2M phosphate buffer pH 8.6 (blank)	534.7	493.3

This data shows that the phosphate eluted the cadaverine, but something else was also eluted that

interfered with the assay.

The interacting unknown may be separated from the cadaverine by washing with another buffer that won't elute the cadaverine. Borate as seen already will not elute cadaverine; thus it was used to wash after the 0.025 M phosphate buffer at pH 6.5. Following two washes of the borate the 0.2M phosphate was used to elute. The borate buffer removed some of the interacting unknown but not all of it. Buffer samples without diamine or tuna were run through the column and assayed. This confirmed that the unknown must have been coming from the tuna because the values were all approximately zero.

The cation exchange column used thus far is more specific for cadaverine than histamine. In other words, the histamine does not stick to the column as tightly as the cadaverine does. Since this is the case, the diamine used was switched to histamine. The column was loaded with 100 uM histamine in buffer. It was washed with 0.1 M borate buffer to try to elute just the histamine off the column. Then, it was washed with 0.2 M borate buffer. The same procedure was performed for the non-spiked tuna column. The first wash seemed to pull off most of the histamine, but a little of the unknown also came off. On the other hand, most of the unknown came off in the 0.2M borate buffer wash.

Future Work:

Presently, the most important goal is to eliminate the interfering unknown. Several simple steps may be taken to inactivate this unknown. The next experiment performed will be an effect of heat study. This, if successful, may be used in conjunction with the column or even better, just the dilution. The effects of heat must also be applied to the diamines, because they may also be affected be heat.