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MUSHROOM TYROSINASE AS A CONTROL MATERIAL FOR PHENOLOXIDASE ASSAYS USED IN THE ASSESSMENT OF CRUSTACEAN "HEALTH"

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ABSTRACT Mushroom tyrosinase (E.C. 1.14.18.1) was evaluated as a control material for use in a plasma prophenoloxidase assay in the American lobster *Homarus americanus* as part of the assay's validation procedure. Reconstituted, lyophilized tyrosinase solution was stable for 72 h at 2–4°C. Percent recoveries were 94%, 120%, 113%, and 48% for "mid-range" activity dilutions and 93%, 94%, 87%, and 38% for "low-range" activity dilutions at 24, 48, 72, and 96 h, respectively. The activity of the reconstituted tyrosinase solution was stable for 4 wk when stored at −80°C, and percentage recoveries, compared to activity of the freshly reconstituted solution, were generally within 15% for both mid-range and "high"-activity dilutions. The between-run coefficients of variation (CVs) were 10.6% for the mid-range and 10.8% for the high-activity dilution over the 4-wk period. Enzyme activity was unstable when stored at −20°C. Mushroom tyrosinase is an acceptable control material for use in assays of phenoloxidase activity. Inclusion of this control material will provide a means to confidently compare results on a day-to-day or run-to-run basis when phenoloxidase assays are used in the assessment of crustacean "health."

KEY WORDS: prophenoloxidase assay, control, tyrosinase, crustacean, health

INTRODUCTION

The prophenoloxidase activating system (PPAS) and its active enzyme product, phenoloxidase (*o*-diphenol:O_2 oxidoreductase E.C. 1.14.18.1) are integral components of the innate defense system in crustaceans (Söderhäll et al. 1996; Söderhäll & Cerenius 1998; Sritunyalucksana & Söderhäll 2000). Dark pigmentation (melaninization) at sites of injury reflects prior generation of phenoloxidase, followed by the spontaneous formation of melanin (Söderhäll et al. 1996; Söderhäll & Cerenius 1998; Sritunyalucksana & Söderhäll 2000). Both the reactive quinone intermediates and the final melanin products formed during this reaction have antimicrobial and protective properties (Söderhäll et al. 1996; Riley 1997).

Determination of the activity of phenoloxidase in either whole hemolymph or hemocyte lysate supernatants has been used to assess the "health" or "immune" status of crustacean species (Hauton et al. 1997; Sritunyalucksana et al. 1999; Rodríguez & Le Moullac 2000). Commercial test kits[1] are available to detect phenoloxidase activity in whole hemolymph samples from shrimp. It is recognized that assays used in the assessment of crustacean health should be standardized (Bachère 2000).

Evaluation and validation of a laboratory test are required to confirm that an assay is performing within its defined parameters before it can be accepted for use in a diagnostic setting (Bellamy & Olexson 2000). This includes determination of the assay's reportable range, precision, accuracy, and reagent stability. Control materials are essential to monitor the performance of the assay (Westgard & Klee 1999). Inclusion of a control with each group of samples permits reliable comparison of the results obtained from samples analyzed during different runs. Ideally, control materials are derived from the same biological source as the samples that are being evaluated.When this is not possible, materials with similar properties can be substituted (Westgard & Klee 1999).

Most phenoloxidase assays are based on (or are modifications of) the method of Horowitz and Shen (Horowitz & Shen 1952; Aspán & Söderhäll 1995). During development of an assay to detect prophenoloxidase activity in plasma of the American lobster, *Homarus americanus* H. Milne Edwards, a control material was required. A purified source of lobster phenoloxidase having consistent activity was not available. Lyophilized mushroom tyrosinase was evaluated as a control material because of its similarities to phenoloxidase and commercial availability (Aspán & Söderhäll 1995).

MATERIALS AND METHODS

Mushroom tyrosinase (E.C. 1.14.18.1) stock solution was prepared by adding 2.5 mL of sodium phosphate buffer (0.2 M Na_2PO_4, pH 7.5) to 13 mg of lyophilized mushroom tyrosinase (Sigma Chemical Company, St. Louis, MO), giving a final concentration of approximately 10,700 units of tyrosinase activity per mL of solution. Serial dilutions of this reconstituted stock solution were used to determine assay linearity, sensitivity, and within-run precision as determined by calculation of coefficient of variation (%CV). A second stock solution, calculated to have similar activity per unit volume, was prepared from a different lot of lyophilized tyrosinase. This second solution was used to conduct refrigerated and frozen stability trials. Eight 150-μL aliquots were placed in 0.5-mL polypropylene microcentrifuge tubes (Fisher Scientific, Canada). Four aliquots were stored at −20°C, four aliquots were stored at −80°C, and the remaining solution was refrigerated (2–4°C), for stability trials.

To complete linearity and precision assessments, serial dilutions at 1:200, 1:400, 1:800, 1:1600, 1:3200, and 1:6400 of the stock solution were made using a 0.2 M sodium phosphate buffer, pH 7.5. Twenty microliters of each dilution was added to individual wells of a flat-bottomed tissue culture plate (Falcon Microtest 3072, Becton Dickinson and Company, U.S.A.). There were five replicates per dilution. Wells to be used as "substrate-free blanks", one well for each dilution, also received 20 μL of the appropriate tyrosinase stock dilution. Twenty microliters of phosphate buffer was placed in wells used as reagent blanks. Eighty microliters of sterile, distilled water was added to all wells using a

[1]Spot On, DiagXotics Inc., Wilton, CT 06897.

multichannel pipette. One hundred microliters of the substrate solution, 3.8 mM dopamine (3-hydroxytyramine) (Sigma Chemical Company), was then added to all wells except for the substrate-free blanks. These wells each received 100 μL of sterile distilled water. Production of the gold-orange quinone intermediate was monitored every 11 sec (wavelength 470 nm) during a 5-min period in an automated spectrophotometer (SpectraMax, Molecular Devices Corporation, U.S.A.) after a 5-sec mixing cycle. The software package SoftMax™ (Molecular Devices Corporation, U.S.A.) was used for calculation of maximum enzyme activity (V_{max}), defined as the rate of maximal change in optical density (OD) of the reaction solution per minute (Δ mOD/min) over a user-defined time interval. The V_{max} used for all further calculations for each dilution was the mean V_{max} of the five replicates. Within-run coefficients of variation [%CV = (standard deviation/mean) × 100] were then calculated (Table 1).

Stability of the refrigerated stock solution was determined by calculating the percentage recovery for two dilutions, identified as having "low" and "mid-range" activities relative to the linear range of the assay, over a period of 4 days. Percent recovery was calculated as: [(measured activity at time "x"/initial activity at reconstitution) × 100] at 24, 48, 72, and 96 h (Table 2). Four replicate wells were used for each dilution.

Long-term frozen stability of the stock solution, stored at −20°C and −80°C, was evaluated by measuring enzyme activity in serial dilutions of a thawed (room temperature, 20–22°C) aliquot every week for 4 wk. Four replicate wells were used for each dilution. Percent recovery calculations were made for the aliquots stored at −20°C and −80°C (Tables 3a and 3b). Between-run (week to week) %CVs were calculated for each dilution of the aliquots stored at −80°C (Table 4).

The refrigerated stability of thawed stock solution, after storage at −80°C, was also evaluated. Enzyme activity was measured at 48 and 72 h post-thawing for two dilutions (mid-range and high activities). Percentage recoveries were calculated by comparing the activity after 2 and 3 days of refrigeration to the activity obtained immediately after thawing (Table 5).

RESULTS

No significant absorbance was detected in the substrate-free blank wells during the initial assays. Substrate-free blanks were not used for the remaining assays. Reagent blanks were used for all assays and also functioned as the plate blanks. The reaction curve was linear during minutes 2–4 of the 5-min monitoring period. V_{max} was determined from this 2-min period. Initially, a gold-orange product developed in all of the reaction wells containing the dopamine substrate and the mushroom tyrosinase. Subsequently, a black product also appeared in the wells. At first, only small amounts of the black product were present; however, with time, the gold-orange product was no longer visible and only the black product could be seen. The amount of this second product seemed proportional to the concentration of enzyme solution in the wells.

TABLE 1.

Sensitivity, linearity, and precision results for reconstituted mushroom tyrosinase.

Dilution	Expected[a] Activity (mOD/min)	Observed Activity[b] (mOD/min)	SD[c]	CV[d] (%)
1:200	—	41.6	0.53	1.3
1:400	20.8	19.6	0.16	0.8
1:800	10.4	9.3	0.39	5.0
1:1600	5.2	5.0	0.32	6.5
1:3200	2.6	1.4	0.20	14.3
1:6400	1.3	0.4	0.23	60.7

[a] Calculated from the 1:200 dilution.
[b] Represents the mean of five replicates.
[c] Standard deviation.
[d] Coefficient of variation [%CV = (standard deviation/mean) × 100].

TABLE 2.

Refrigerated (2–4°C) stability of reconstituted mushroom tyrosinase.

	Initial Activity[b] (mOD/min)	Percent Recovery (%)[a]			
		1 day	2 days	3 days	4 days
Dilution no. 1	22.4	94	120	113	48
Dilution no. 2	12.3	93	94	87	38

[a] Percent recovery = (measured activity/initial activity) × 100.
[b] Represents the mean of four replicates.

The catechol oxidase activity of mushroom tyrosinase, as measured in this assay system, was linear, and had acceptable precision results, from 5.0 to 41.6 mOD/min when evaluated by serial dilution (R^2 = 0.99) (Table 1, Fig. 1). Using the activity of the 1:200 dilution as a starting point, the expected and observed (in parentheses) activities (mOD/min) for each further dilution were 20.8 (19.6) for the 1:400 dilution, 10.4 (9.3) for the 1:800 dilution, 5.2 (5.0) for the 1:1600 dilution, 2.6 (1.4) for the 1:3200 dilution, and 1.3 (0.4) for the 1:6400 dilution. The within-run CVs (n = 5 replicates), for each dilution were 1.3% for the 1:200 dilution, 0.8% for the 1:400 dilution, 5.0% for the 1:800 dilution, 6.5% for the 1:1600 dilution, 14.3% for the 1:3200 dilution, and 60.7% for the 1:6400 dilution (Table 1).

The stability of the freshly reconstituted reagent was acceptable (i.e., generally within 15% of the initial values) for up to 72 h after refrigeration, but decreased to an average of 43% of initial activity by 96 h (Table 2). Percent recoveries for a mid-range dilution at 24, 48, 72, and 96 h were 94%, 120%, 113%, and 48%, respectively. Percent recoveries for a low-activity dilution at 24, 48, 72, and 96 h were 93%, 94%, 87%, and 38%, respectively (Table 2).

The stock solution was unstable when stored at −20°C. The enzyme activity, calculated as a mean of all dilutions, decreased to 37%, 28%, 27%, and 21% of the initial activity of the freshly reconstituted reagent by weeks 1, 2, 3, and 4, respectively (Table 3a).

The stock solution was stable when stored at −80°C. The percent recoveries, average of mid-range (dilution no. 2) and high- (dilution no. 1) activity dilutions, by week, were 87% for week 1, 99% for week 2, 116% for week 3, and 106% for week 4 (Table 3b). The between-run CVs, over the 4-wk period, were 10.6% for the mid-range dilution and 10.8% for the high-activity dilution (Table 4). The refrigerated stability of the thawed stock solution was generally satisfactory for weeks 1–3, but tended to deteriorate by week 4 of storage at −80°C (Table 5).

DISCUSSION

The value of any assay resides in its ability to consistently provide accurate and precise results. An incorrect result can be

TABLE 3a.

Frozen stability of mushroom tyrosinase stock solution stored at −20°C for 4 weeks.

Dilution	Initial Activity[b] (mOD/min)	Percent Recovery (%)[a]			
		1 wk	2 wk	3 wk	4 wk
No. 1	33.4	37	28	26	22
No. 2	17.0	37	28	28	19
Mean		37	28	27	21

[a] Percent recovery = (measured activity/initial activity) × 100.
[b] Represents the mean of four replicates.

worse than no result at all. Assay validation includes quality control steps used to ensure that diagnostic tests perform within predetermined specifications. Control materials are used to monitor the assay's performance on a run-to-run basis. Ideally, such materials are derived from the same species as the samples to be analyzed; however, this is not always possible. Steps in assay validation usually include (but are not limited to): determination of the assay's reportable range; evaluation of assay precision by calculating both within-run and between-run coefficients of variation (%CV = standard deviation/mean × 100); and, determining the stability of the assay reagents and the sample under different storage conditions (Bellamy & Olexson 2000).

Phenoloxidase (*o*-diphenol:O_2 oxidoreductase E.C. 1.14.18.1) assays have been used to investigate and assess the health status of a variety of crustaceans in both research and commercial settings (Sritunyalucksana et al. 1999; Rodríguez & Le Moullac 2000). A stable source of enzyme to use as a control material was required in the development of a plasma prophenoloxidase assay for use in *H. americanus*. A commercial source of purified lobster (*H. americanus*) phenoloxidase is not available. Lobster hemocyte lysate supernatant preparations represented a highly concentrated source of prophenoloxidase; however, these were unsuitable due to their poor long-term storage characteristics and the inherent, inconsistent activity between preparations (unpubl. results, ALB).

Mushroom tyrosinase (E.C. 1.14.18.1) was evaluated as a potential alternative because of its similarity to phenoloxidase. Both enzymes have cresolase (monophenol monooxygenase) and catechol oxidase (polyphenol oxidase) activity (Aspán & Söderhäll 1995). Determination of the enzyme's copper content and location of conserved histidine residues indicates that arthropod prophenoloxidase belongs to the tyrosinase group of enzymes found in bacteria, fungi, and plants (Aspán & Söderhäll 1995). The reported long-term stability of frozen, reconstituted mushroom tyrosinase

TABLE 3b.

Frozen stability of mushroom tyrosinase stock solution stored at −80°C for 4 weeks.

Dilution	Initial Activity[b] (mOD/min)	Percent Recovery (%)[a]			
		1 wk	2 wk	3 wk	4 wk
No. 1	33.4	85	93	112	107
No. 2	17.0	89	105	119	105
Mean		87	99	116	106

[a] Percent recovery = (measured activity/initial activity) × 100.
[b] Represents the mean of four replicates.

TABLE 4.

Weekly between-run coefficients of variation (CV) for mushroom tyrosinase activity when stored at −80°C.

Weeks at −80°C	Mean[a] Activity (mOD/min)	
	Dilution No. 1	Dilution No. 2
0	33.4	17.0
1	28.4	15.1
2	31.1	17.7
3	37.2	20.2
4	35.7	17.9
Mean	33.1	17.6
SD[b]	3.5	1.9
CV[c] (%)	10.6	10.8

[a] n = 4 replicates.
[b] Standard deviation.
[c] Coefficient of variation = (mean/standard deviation) × 100.

and its commercial availability made this enzyme an attractive alternative (Kertesz & Zito 1965).

Mushroom tyrosinase was readily soluble in the 0.2 M sodium phosphate buffer (pH 7.5) used in this assay system. Other studies have used a sodium cacodylate-based buffer. Sodium cacodylate is listed as a hazardous material and is a recognized carcinogen (Sigma-Aldrich 2001). The sodium phosphate buffer used herein presents minimal hazards, is readily available, and was compatible with this assay system. L-DOPA (3,4-dihydroxyphenyl-L-alanine), a commonly used substrate in phenoloxidase assays, was not compatible with the phosphate buffer. Dopamine (3-hydroxytyramine) was chosen as an alternate catechol. Dopamine has been identified as a preferred substrate for phenoloxidase from *Manduca sexta* (Sugumaran et al. 1999). This may also be true for other invertebrates. Mushroom tyrosinase converted the dopamine substrate to an orange-gold intermediate product, presumably the *o*-quinone, 4-(2-aminoethyl)-1,2-benzoquinone. This product was eventually replaced by a black precipitate (melanin), which is the expected result of activation of the PPAS *in vivo* (Söderhäll et al. 1996; Söderhäll & Cerenius 1998; Sritunyalucksana & Söderhäll

TABLE 5.

Refrigerated stability of thawed mushroom tyrosinase stock solution stored at −80°C.

Dilution	Weeks at −80°C	Initial activity[b] (mOD/min)	Percent recovery (%)[a]	
			2 days	3 days
No. 1	0	33.4	nd[c]	nd
	1	28.4	110	135
	2	31.1	106	110
	3	37.2	106	102
	4	36.7	85	75
No. 2	0	17.0	nd	nd
	1	15.1	106	139
	2	17.7	86	103
	3	20.2	104	97
	4	17.9	84	89

[a] Percent recovery = (measured activity/initial activity) × 100.
[b] Represents the mean of four replicates.
[c] Not done.

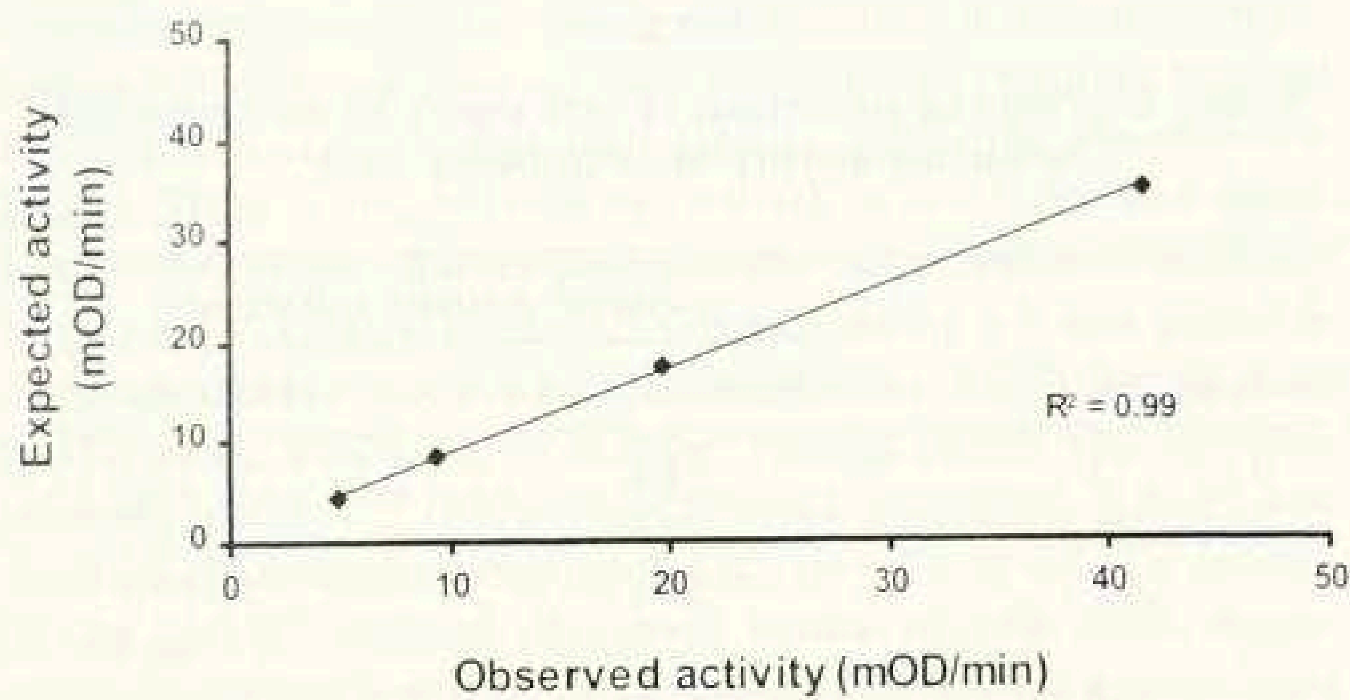

Figure 1. Linearity of catechol oxidase activity of reconstituted mushroom tyrosinase determined by serial dilution.

2000). An identical series of products is observed when either lobster hemocyte lysate supernatant or lobster plasma, after activation with trypsin, is used as the enzyme source for the assay.

The reconstituted tyrosinase solution was stable, when refrigerated, for up to 3 days as indicated by the percentage recovery studies (Table 2). Tyrosinase activity was stable for up to 4 wk when the reconstituted solution was stored at −80°C. Reconstituted mushroom tyrosinase was unstable when stored at −20°C, in contrast to a previous report (Kertesz & Zito 1965). The discrepant results found in this study may reflect differences in the buffers used for reconstitution. Overall, the refrigerated and frozen (−80°C) stability of reconstituted mushroom tyrosinase was considered acceptable for its use as an assay control material.

Ideally, control materials contain a predetermined amount of the enzyme being measured and are incorporated in a material (matrix) similar to the matrix of the sample to be analyzed. In this study, mushroom tyrosinase was reconstituted in a phosphate buffer. This would be the optimal situation when phenoloxidase activity is being measured in hemocyte lysate supernatants where a similar buffer had been used to prepare the lysates. Similarly, incorporation of the mushroom tyrosinase into whole hemolymph or plasma collected from normal, apparently healthy animals would be preferable when phenoloxidase activity is being measured in these types of samples. This was not evaluated in this study. When reconstituted in a sodium phosphate buffer, mushroom tyrosinase is a suitable control material for use in phenoloxidase assays, providing assurance that the assay is working correctly and permitting comparison of within-laboratory sample results on a run-to-run or day-to-day basis.

LITERATURE CITED

Aspán, A. & K. Söderhäll. 1995. The prophenoloxidase activating system in invertebrates: Assays of the prophenoloxidase activating enzyme (a serine proteinase) and phenoloxidase. In: J. S. Stolen, T. C. Fletcher, S. A. Smith, J. T. Zelikoff, S. L. Kaattari, R. S. Anderson, K. Söderhäll & B. A. Weeks-Perkins, editors. Techniques in fish immunology—4. Fair Haven, New Jersey: SOS Publications. pp. 161–170.

Bachère, E. 2000. Introduction: Shrimp immunity and disease control. *Aquaculture* 191:3–11.

Bellamy, J. E. C. & D. W. Olexson. 2000. Evaluating laboratory procedures. In: J. E. C. Bellamy & D. W. Olexson, editors. Quality assurance handbook for veterinary laboratories. Ames: Iowa State University Press. pp. 61–77.

Hauton, C., J. A. Williams & L. E. Hawkins. 1997. The effects of a live *in vivo* pathogenic infection on aspects of the immunocompetence of the common shore crab, *Carcinus maenas* (L.). *J. Exp. Mar. Biol. Ecol.* 211:115–128.

Horowitz, N. H. & S. C. Shen. 1952. *Neurospora* tyrosinase. *J. Biol. Chem.* 197:549–557.

Kertesz, D. & R. Zito. 1965. Mushroom polyphenol oxidase I. Purification and general properties. *Biochim. Biophys. Acta* 96:447–462.

Riley, P. A. 1997. Molecules in focus: Melanin. *Int. J. Biochem. Cell Biol.* 29:1235–1239.

Rodríguez, J. & G. Le Moullac. 2000. State of the art of immunological tools and health control of penaeid shrimp. *Aquaculture* 191:109–119.

Sigma-Aldrich Canada Ltd. 2001. Cacodylic acid sodium salt trihydrate material safety data sheet. Oakville: Sigma-Aldrich Canada Ltd.

Söderhäll, K & L. Cerenius. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* 10:23–28.

Söderhäll, K., L. Cerenius & M. W. Johansson. 1996. The prophenoloxidase activating system in invertebrates. In: K. Söderhäll, S. Iwanaga & G. R. Vasta, editors. New directions in invertebrate immunology. Fair Haven: SOS Publications. pp. 229–253.

Sritunyalucksana, K. & K. Söderhäll. 2000. The proPO and clotting system in crustaceans. *Aquaculture* 191:53–59.

Sritunyalucksana, K., P. Sithisarn, B. Withayachymnarnkul & T. W. Flegel. 1999. Activation of prophenoloxidase, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants. *Fish Shellfish Immunol.* 9:21–30.

Sugumaran, M., R. Duggaraju, F. Generozova & S. Ito. 1999. Insect melanogenesis. II. Inability of *Manduca* phenoloxidase to act on 5,6-dihydroxyindole-2-carboxylic acid. *Pigment Cell Res.* 12:118–125.

Westgard, J. O. & G. G. Klee. 1999. Quality management. In: C. A. Burtis & E. R. Ashwood, editors. Tietz textbook of clinical chemistry, 3rd ed. Philadelphia: W.B. Saunders. pp. 384–418.