ANALYTICAL BIOCHEMISTRY 76, 374–376 (1976)

An Improved Color Reagent for Use in Barrett's Assay of Cathepsin B

Barrett (1) described a new, sensitive assay procedure for cathepsin B (EC 3.4.22.1) (previously called cathepsin B1) in which the substrate benzoyl-DL-arginine 2-naphthylamide is cleaved, and the free naphthylamine is coupled with a diazonium salt, fast garnet, to yield a red color which is quantitated as $A_{520\ nm}$. The enzyme requires the presence of a thiol compound for activity, but diazonium salts are inactivated by these compounds (presumably by a displacement reaction), and it was therefore necessary to formulate a special coupling reagent which contained a mercurial compound to protect the diazonium salt by blocking the thiol reagent while stopping the enzymic reaction. The thiol-blocking reagent used was 4-chloromercuribenzoate, but the limited solubility of this reagent had two disadvantages. First, in order to keep the mercurial in solution, the pH of the color reagent was made 6.0. This seriously decreased the stability of the diazonium salt, which is completely stable only at pH 4 and below. Second, the amount of the mercurial that could be kept in solution was equivalent to only 5 mM thiol activator, whereas one would sometimes wish to use more. An additional disadvantage of the original reagent was that it was not suitable for use with dithiothreitol as activator.

The new color reagent described here contains a much more soluble mercurial compound, which overcomes these problems. Also, the diazonium salt is prepared directly by diazotization of the amine, which has been found to give better results than the use of commercial preparations of stabilized fast garnet. Both changes decrease the cost of the reagent.

PREPARATION OF THE REAGENT

The color reagent is prepared from two stock solutions made up as follows.

Amine stock solution. Take 225 mg of 4-amino-2′,3-dimethylazobenzene (a product of Merck-Schuchardt, available from Cambrian Chemicals, Ltd., Suffolk House, George Street, Croydon CR9 3QL, England), crush to a powder, and dissolve in 50 ml of ethanol. Add 30 ml of 1 M HCl, with stirring, dilute to 100 ml with water, and store at 4°C.

Mersalyl–Brij reagent. Place 2.43 g of mersalyl acid (i.e., 2-{[3-(hydroxymercuri)-2-methoxypropyl]carbamoyl}phenoxyacetic acid, available from Sigma Chemical Co.) in a beaker containing a stirring bar, and dissolve

TABLE 1

COLOR YIELDS FOR FAST GARNET–2-NAPHTHYLAMINE REACTION MIXTURES[a]

Thiol concentration (mg-equiv/liter)	3	7.5	15
Mersalyl concentration (mM)	5	10	20
Thiol compound	Color yields (%)		
None	100	100	100
Glutathione	101	101	99
Mercaptoethanol	100	99	88
Cysteine	102	100	99
Dithiothreitol	101	99	107
Mercaptoethylamine	100	101	101

[a] Interference by thiol reagents was suppressed by mersalyl acid. Values are ΔA_{520} calculated as the mean of three tests less a blank, expressed as a percentage of the control value with no thiol compound. The absolute ΔA_{520} values of the control tubes averaged 0.746.

the solid with 60 ml of 0.5 M NaOH. Add 9.3 g of disodium EDTA and make up to about 450 ml with water. Allow the EDTA to dissolve, then adjust the pH to 4.0 by addition of 1 M HCl, with stirring. Make up and add 500 ml of 4% (w/v) aqueous Brij 35 (polyexyethylene lauryl ether, from BDH Chemicals Ltd., Poole, England). Store the reagent in a brown bottle at room temperature.

Prepared as described, the mersalyl–Brij reagent contains 5 mM mersalyl acid, which is ample for routine use, but when high concentrations of thiols are present, the quantity of mersalyl acid can be increased to 10 or 20 mM with no other change (see below).

Color reagent. The color reagent is prepared as follows. Place 1.0 ml of amine stock solution in a test tube standing in a beaker of ice and water. Add 100 μl of 0.2 M $NaNO_2$, mix, and leave at least 5 min, then dilute to 100 ml with the mersalyl–Brij reagent. The reagent is kept cold and used the same day.

RESULTS AND DISCUSSION

The new color reagent is used exactly as was that described previously (1). In order to test the effectiveness of the reagent, the color reaction was applied to solutions containing a standard amount of 2-naphthylamine together with various amounts of several thiol compounds.

Three series of tubes were set up. Each tube contained 0.5 ml of 1% ethanol only (blanks) or with 0.1 μmol of 2-naphthylamine–HCl (equivalent to 14.3 μg of free base). To each was then added 1.5 ml of 0.1 M potassium sodium phosphate buffer, pH 6.0, containing 1.33 mM disodium EDTA (1) in which had been dissolved glutathione, mercaptoethanol, cysteine, dithiothreitol, or mercaptoethylamine–HCl. Two milliliters of

color reagent was then added to each tube. In the first series of tubes, the concentration of thiol groups was 3 mg-equiv/liter (i.e., 3 mM monothiols or 1.5 mM dithiothreitol) before the introduction of the color reagent, which was the standard reagent containing 5 mM mersalyl acid. Higher concentrations of thiol were used with two- or fourfold increased concentrations of mersalyl salt, as indicated in Table 1, which shows the color yields obtained.

It can be seen that there was complete elimination of interference by glutathione, cysteine, and mercaptoethylamine even at 15 mM concentration. At the equivalent concentration, mercaptoethanol and dithiothreitol showed some interference, but this was not apparent at half the concentration. Since complete activation of purified human cathepsin B (2) is achieved with 1 mM dithiothreitol or 2 mM cysteine (1,2), the possibility of using much higher concentrations of activators is largely academic, for this enzyme, and the color reagent containing 5 mM mersalyl acid are perfectly suitable for routine use. Because of its slow autoxidation and other advantages (3) dithiothreitol (1 mM in the incubation mixture) now may be the activator of choice.

We have found commercial samples of fast garnet salt stabilized with $ZnCl_2$ or fluoroborate to be grossly impure, so that $A_{520\ nm}$ values for reagent blanks were distinctly higher than with the present reagent. Also, the poor solubility of the commercial products made them inconvenient to use.

The sensitivity of the present color reagent is indicated by the A_{520} value of 0.746 obtained with 14.3 μg of naphthylamine per tube (Table 1). The difference between this and values of about 0.227 obtained with 20 μg/tube by a recently described extraction method (4) is attributable to the stronger color of the fast garnet–2-naphthylamine product as compared with that of tetrazotized *o*-dianisidine (1) and also to greater dilution in the latter method. We have found that still greater sensitivity can be obtained in the assay of cathepsin B by use of the new substrates, such as benzyloxycarbonyl-diarginine 2-naphthylamide (5), in place of benzoyl-DL-arginine 2-naphthylamide, in conjunction with the new color reagent.

REFERENCES

1. Barrett, A. J. (1972) *Anal. Biochem.* **47,** 280–293.
2. Barrett, A. J. (1973) *Biochem. J.* **131,** 809–822.
3. Cleland, W. W. (1964) *Biochemistry* **3,** 480–482.
4. Doebber, T. W., and Miller, L. L. (1976) *Anal. Biochem.* **70,** 39–44.
5. McDonald, J. K., and Ellis, S. (1975) *Life Sci.* **17,** 1269–1276.

ALAN J. BARRETT

Strangeways Research Laboratory
Cambridge CB1 4RN
England

Received April 20, 1976; accepted July 1, 1976