N-D-Biotinyl-7-amino-4-methylcoumarin as a Novel Fluorigenic Substrate for the Determination of Biotinidase Activity

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A new fluorigenic substrate for the determination of biotinidase, N-D-biotinyl-7-amino-4-methylcoumarin has been synthesized by using BOP reagent. This substrate was used for measuring the enzyme activity in plasma of normal and biotinidase deficient patients. The methodology used here should be applicable to other biological fluids and homogenates to detect biotinidase deficiency.

Biotinidase (E.C.3.5.1.12) liberates biotin from biocytin and other compounds to reuse this vitamin. Deficiency of this enzyme may develop the characteristic findings such as alopecia, ataxia and others. To perform early treatment with large doses of biotin, various detection methods of biotinidase activity have been developed. Besides detection by microbiological methods[1], the biotinidase activity can be determined with radiochemical,[2,3] colorimetric,[4,5] and fluorometric methods.[6-8] For quick and sensitive determination the fluorometric method has an advantage but the fluorigenic substrate previously reported, namely biotynyl-6-aminoquinoline, has not accomplished routine application for clinical analysis, mainly due to its low solubility, higher pH-dependency and rather long lag phase observed.

We have recently synthesized new fluorigenic substrate for biotinidase, N-D-biotinyl-7-amino-4-methylcoumarin, and applied for plasma of biotinidase deficient patients.

To a chilled solution of D-biotin (244 mg, 1.0 mmol) (Tokyo Kasei, Tokyo) and 7-amino-4-methylcoumarin (175 mg, 1.0 mmol) (Tokyo Kasei) in dimethylformamide (DMF) (5 ml) (Wako Pure Chemicals, Osaka) was added BOP reagent (benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexa-fluorophosphate) (530 mg, 1.2 mmol) (Wako Pure Chemicals) and triethylamine (0.21 ml, 1.5 mmol) (Wako Pure Chemicals). The reaction mixture was stirred at 0 ℃ overnight. Precipitate (unknown by-product) was filtered, and the filtrate was evaporated in vacuo. The residues were dissolved in $CHCl_3/CH_3OH$ (5/1, 3 ml) (Wako Pure Chemicals) and subjected to the column (2 x 24 cm) of silica gel 60 (E.Merck, Darmstadt, Germany) and eluted with $CH\,Cl_3$ /CH_3OH (95/5). Yield 80 mg (20%). Soluble to DMF and dimethyl sulfoxide. TLC ($CHCl_3/CH_3OH$ (9/1)) 0.35. 1H-NMR (400 MHz, DMSO-d_6) δ 1.35-1.70 (m, 6H), 2.35-2.40 (m, 5H), 2.58 (d, 2H, J = 12.6 Hz), 2.83(dd, 1H, J= 5.2, 12.6 Hz), 3.10-3.18 (m, 1H), 4.12-4.16 (m, 1H), 4.29-4.33 (m, 1H), 6.26 (s, 1H), 6.37 (s, 1H), 6.44 (s, 1H), 7.48 (dd, 1H, J= 2.0, 9.0 Hz), 7.71 (d, 1H, J= 9.0 Hz), 7.77(d, 1H, J= 2.0Hz), 10.33 (s, 1H). The formation of the aromatic amide bond was detected by the peak at δ = 10.33. FAM-MS m/z 402 $(M+H)^+$.

The obtained substrate has been used for determination of biotinidase in plasma samples of normal and 2 known patients with respectively total and partial biotinidase deficiency. To promote the solubility of the substrate, we have used β-cyclodextrin (Sigma, St.Louis, USA).[9] Fifteen micro litters of 0.2 mM of the substrate in 3% β-cyclodextrin was incubated with 5 μl of 1 M phosphate-citrate buffer (pH 5.5) containing 20 mM EDTA (Jannsen Chimica, Beerse, Belgium), 5 μl of 120 mM mercaptoethanol (Jansen Chimica, Beerse, Belgium), and 10 μl of acidified[10] plasma for 10 to 20 min. Termination of the reaction was done with 1 ml of 1 M formiate buffer (pH 3) and the liberated 7-amino-4-methylcoumarin was measured in Turner fluorimeter model 111 at excitation 360 nm and emission 460 nm. Although these wavelengthes are not very optimal for 7-amino-4-methyl-coumarin determination, we have chosen them because they can be used in the determination for other enzymes with fluorigenic substrates, such as various derivatives of metheylumbelliferone, to provide detections of a series of enzyme deficiency samples.

The activity in normal plasma samples was 4.8 mU (unit is defined as nanomoles of substrate hydrolyzed per min.) per ml, while 0 and 1.0 mU per ml in one deficient and one partially deficient patient, respectively. These values are well corresponding to the values determined by the standard clinical method[4]; 7.1±2.7, 1.6±0.4, and 0.1 (nmoles of *p*-aminobenzoate liberated per min per ml from N-biotinyl-*p*-aminobenzoic acid), respectively. The optimum pH and apparent Km value were 5.5 and 0.03 mM, respectively. Biotinidase is rather an unstable enzyme at 37 ℃, since residual activity with or without addition of mercaptoethanol (up to 10 mM) was only about 10% after 3 h. At 23 ℃, however, the activity was conserved during 24 h. The addition of mercaptoethanol on the

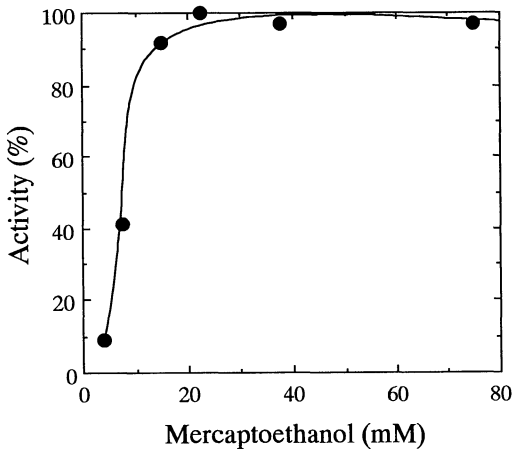

Figure 1. Effect of mercaptoethanol addition on the restoration of the enzyme activity from the frozen plasma samples, stored at -30 ℃.

measurement of the enzyme activity in samples kept frozen restored enzyme activity (Figure 1.) and even after storage of samples at -30 ℃ we noted that the activity could be restored. Addition of thiol (optimally active at about 20 mM) is mandatory since the activity in frozen samples was lost but could be fully restored on addition of reducing groups afterward. These findings are related to the indications of the thiol-type character of human serum biotinidase by other authors.[11-13]

The present substrate and methodology proved to be exceedingly easy and the enzyme reaction itself, fixation and reading of fluorescence could be done in the same small reaction tube. The methodology used here should be applicable to other biological fluids and homogenates and can find applications in screening and detection of patients with biotinidase deficiency.

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References and Notes

1 H.R.Skeggs and L.D.Wright, *Proc.Soc.Exp.Biol.*, **56**, 95 (1944).
2 B.Wolf and J.S.McVoy, *Clin.Chim.Acta*, **135**, 275 (1983).
3 L.E.Thuy, B.Zielinska, L.Sweetman, and W.L.Nyhan, *Ann. N.Y.Acad.Sci.*, **1985**, 447.
4 J.Knappe, W.Brunner, and K.Biederbick, *Biochem.Z.*, **338**, 599 (1963).
5 J.Knappe, W.Brommer, and K.Biederbick, *Biochem.Biophys. Acta*, **1074**, 443 (1991).
6 K.Hayakawa and J.Oizumi, *J.Chromatgraphy*, **35**, 831 (1989).
7 H.Wastell, G.Dule, and K.Bartlett, *Anal.Biochem.*, **140**, 69 (1984).
8 H.Ebrahim and K.Dakshinamurti, *Anal.Biochem.*, **154**, 282 (1986).
9 During our experiments, a review on an independent study of using enzyme reactions in the presence of cyclodxtrins for biosensor was published: J.H.Luong, R.S.Brown, K.B.Male, M.V. Cattaneo, and S. Zhao, *Trends in Biotechnology*, **13**, 457 (1995).
10 W.R.Den Tandt, E.Lassila, and M.Philippart, *J.Lab.Clin.Med.*, **83**, 403 (1974).
11 D.V.Craft, N.H.Goss, N.Chandramouli, and H.G.Wood, *Biochemistry*, **24**, 2471 (1985).
12 K.Hayakawa and J.Oizumi, *J.Biochem.(Tokyo)*, **103**, 773 (1988).
13 J.Chauhan and K.Dakshinamurti, *J.Biol.Chem.*, **261**, 4268 (1986).