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Methyl and phenyl esters and thioesters of carboxylic acids as surrogate substrates for microassay of proteinase K esterase activity

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Abstract The development of a microassay for proteinase K esterase activity with carboxylic acid esters is reported using novel substrates of the general formula R-C(O)-XR´. Highest rates of hydrolysis have been obtained with the O-phenyl esters $CH_3(CH_2)_{n=1-2}$-S-$(CH_2)_{n=1-2}$C(O)-O-phenyl and their thioester analogs in studies where *R, X* and *R´* have been varied. The phenol release has been measured with 4-aminoantipyrine and potassium ferricyanide to determine the rates of O-phenyl ester hydrolyses. Thioester hydrolyses have been monitored continuously with 5,5´-dithio-bis (2-nitrobenzoic acid).

Introduction

The fungal enzyme proteinase K (EC 3.4.21.14) is used to inactivate nucleases during isolation of RNA and DNA [1], and to solubilize membrane-bound proteins such as acetylcholinesterase from the electric organ of *Torpedo* [2]. The enzyme catalyzes the hydrolysis of both peptide and ester bonds [3, 4]. Esters of N-acylated amino acids and peptides have been used for the assay of its esterase activity [3, 4]. The continuing interest in the evolution and functional features of proteinase K [5] prompted us to examine a series of carboxylic acid esters as possible surrogate substrates to esters of amino acids and peptides. The development of a new microassay for proteinase K esterase activity is reported based on the hydrolysis of carboxylic acid esters and thioesters.

Experimental

Reagents. Proteinase K (enzyme from *Tritirachium album*) was purchased from Sigma Chemical Co., Mo. Enzyme purity was examined by isoelectric focusing (Rotofor, BioRad, CA). More than 98% of the catalytic activity for phenyl valerate and hemoglobin [6] hydrolysis was recovered in a protein fraction at pI > 9.0 (the reported pI is 8.9 [3]). Stock solutions of the enzyme in 0.05 mol/l Tris buffer, pH 7.5 (25 mg/ml) could be stored without significant loss of esterase activity for at least 3 weeks at –20° C. This stock solution was diluted 1 : 50 (v/v) with the Tris buffer prior to use.

Esters and thioesters of the general formula RC(O)XR´ with varying *R, X* and *R´* were synthesized earlier for carboxylesterases, juvenile hormone esterase and neuropathy esterase [7, 8]. Stock solutions of substrates (30 mmol/l) were prepared in dimethylsulfoxide for phenyl esters and methanol for methyl and phenyl thioesters.

Enzyme assays. The rate of thioester hydrolysis was measured spectrophotometrically at 405 nm by modifying the Ellman assay of thiols [9] for a microassay in 96-well microtiter plates (Falcon, Lincoln Park, NJ) [7]. Proteinase K (10 μg in 20 μl) was added to a mixture of Tris-HCl buffer, pH 8 (276 μl), 57 mmol/l 5,5´-dithio-bis (2-nitrobenzoic acid) ethanolic solution (2 μl) and 30 mmol/l substrate (2 μl). The reaction mixture was incubated at 37° C. The absorbance was measured continuously at 405 nm with an optical 96-well Plate Reader (Bio-Tek Instrument, VT). Thiol concentration was calculated from a calibration curve prepared with glutathione. The hydrolytic rates were computed by the ENZFITTER program for linear regression [10].

Hydrolysis of phenyl esters was determined spectrophotometrically by measuring the released phenol with 4-aminoantipyrine and potassium ferricyanide [11]. The Tris buffer (139 μl) and 30 mmol/l substrate (1 μl) were mixed with proteinase K (5 μg in 10 μl) in a well of a 96-well microtiter plate. The reaction mixture was incubated for 15 min at 37° C. The enzymatic hydrolysis was terminated by adding 50 μl of a solution containing 2 mmol/l 4-aminoantipyrine and 350 mmol/l SDS, followed by 50 μl of 12 mmol/l potassium ferricyanide as the colorimetric reagent. The absorbance was measured at 490 nm 7 min later. The phenol concentration was calculated from a calibration curve prepared with the phenol standards. The rate of phenyl valerate hydrolysis was constant up to 15 min. Response to varying amounts of proteinase K was linear up to 0.00333% enzyme preparation (5.0 μg proteinase K in 150 μl of a reaction mixture). The apparent Michaelis-Menten parameters K_m and V_{max} were determined for phenyl valerate with initial substrate concentrations of 0.49–4.9 mmol/l. The data were computed by the ENZFITTER program [10].

Results and discussion

Changes in *R, R´* and *X* of esters and thioesters R-C(O)-XR´ had profound effects on the rate of their hydrolysis catalyzed by proteinase K (Table 1). Four structural modifications were considered: varying the length of the aliphatic *R;* insertion of a heteroatom into *R;* substituting sulfur or oxygen for *X* and phenyl or methyl for *R´*.

The length of the aliphatic chain *R* was critical for hydrolysis of S-methyl thioesters (series 1 substrates, Table 1). Thioester with R = C_7 (1.2) was the only one that was hydrolyzed at a measurable rate. Hydrolysis was negligible for R = C_5 (substrate 1.1) and diminished to zero with an increasing number of carbons (substrates 1.3–1.8). Similarly, the hydrolysis of thioesters with beta sulfur substituted into *R* diminished with an increasing length of the carbon chain (substrate 1.9 vs 1.10–1.12).

Heteroatom substitution within the aliphatic chain of S-methyl thioesters affected the rates of hydrolyses dependent on

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Table 1 Proteinase K esterase activity[a]

Compound		Activity[b, c, d] nmol · min^{-1} · mg^{-1}
No.	R	
1. R-C(O)-S-CH_3		
1.1	$CH_3(CH_2)_4$	0[e]
1.2	$CH_3(CH_2)_6$	4.4 ± 0.3
1.3–1.8	$CH_3(CH_2)_{7-13}$	0
1.9	$CH_3(CH_2)_4SCH_2$	9.7 ± 1.8
1.10–1.12	$CH_3(CH_2)_{5-7}SCH_2$	0
1.13	$CH_3(CH_2)_5NHCH_2$	0
1.14	$CH_3(CH_2)_5OCH_2$	2.9 ± 2.3
1.15	$CH_3(CH_2)_4S(CH_2)_2$	14.9 ± 4.1
2. R-C(O)-S-phenyl		
2.1	$CH_3(CH_2)_4$	12.7
2.2	$CH_3(CH_2)_3O$	0
2.3	$CH_3(CH_2)_2OCH_2$	30.2 ± 0.8
2.4	$CH_3(CH_2)_2SCH_2$	59.0 ± 0.2
2.5	$CH_3CH_2O(CH_2)_2$	9.1
2.6	$CH_3CH_2S(CH_2)_2$	37.4
3. R-C(O)-O-phenyl		
3.1	$CH_3(CH_2)_3$	43.3
3.2	$CH_3(CH_2)_4$	35.8 ± 2.7
3.3	$CH_3(CH_2)_3S$	0.6 ± 0.3
3.4	$CH_3(CH_2)_3O$	18.4 ± 2.4
3.5	$CH_3(CH_2)_2OCH_2$	54.4 ± 6.3
3.6	$CH_3(CH_2)_2SCH_2$	89.9 ± 13.4
3.7	$CH_3CH_2O(CH_2)_2$	28.4 ± 15.3
3.8	$CH_3CH_2S(CH_2)_2$	95.9 ± 19.7

[a] Proteolytic activity assayed with hemoglobin [6] was 4.8 ± 0.8 μmol · min^{-1} · mg^{-1}

[b] Arithmetic average ± range of two independent determinations of triplicate assays for substrates of No. 1 and 2 series. Standard error of the measurements was 4–12% except for low activities with substrates 1.2 and 1.14 (S.E. 19–39%)

[c] Activity ± S.D. of three independent experiments measured in triplicates for series No. 3 substrates

[d] The rates of substrate spontaneous hydrolyses deducted

[e] Low activity detectable after 24 h

the type of heteroatom and the location of the substitution. In the beta substituted series, the oxygen substituted substrate (1.14) was hydrolyzed at a low rate but hydrolysis was abolished by sulfur (1.10) and amino (1.13) substitutions. The only beta sulfur substituted S-methyl thioester that was hydrolyzed (1.9) was the one with the same number of atoms in *R* as substrate 1.2 (substrate 1.9). The gamma sulfur substitution (substrate 1.15) had the largest effect on the rate of hydrolysis. A similar trend of enzymatic rates of hydrolysis has been observed with several other esterases [7].

In the O-phenyl ester and S-phenyl thioester series (series 2 and 3, Table 1), the beta and gamma sulfur substituted substrates were hydrolyzed at a higher rate than their corresponding oxygen analogs (substrate 2.4 vs 2.3; 2.6 vs 2.5; 3.6 vs 3.5 and 3.8 vs 3.7). Thioesters with beta substituted heteroatoms were hydrolyzed at higher rates than their corresponding gamma analogs (substrate 2.3 vs 2.5 and 2.4 vs 2.6). There were either none or only moderate differences between the hydrolytic rates of the beta and gamma substituted phenol esters (3.5 vs 3.7 and 3.6 vs 3.8). The alpha substituted substrates (2.2, 3.3, 3.4) were hydrolyzed at lower rate as compared to phenyl and thiophenyl

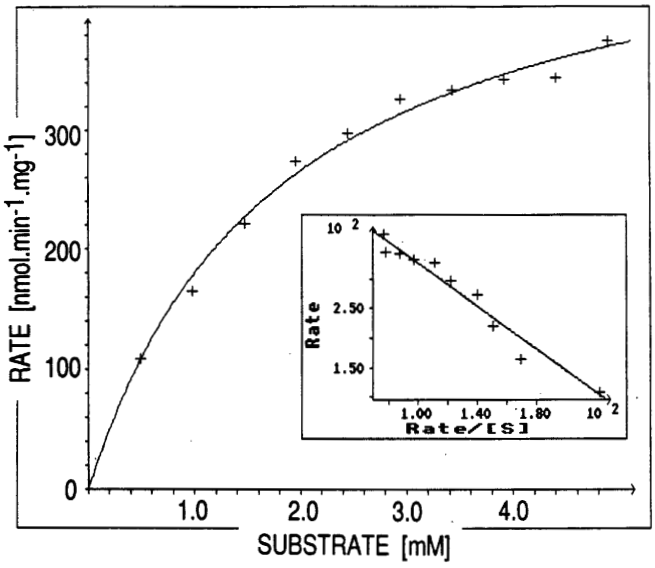

Fig. 1 Changes in rate of phenyl valerate hydrolysis with varying substrate concentrations. The values are an arithmetic average of three measurements. Apparent K_m and V_{max}, computed by (10), are 1.8 ± 0.2 (S.D.) mmol/l and 509 ± 20 (S.D.) nmol · (mg protein)$^{-1}$ · min^{-1}, respectively. Inset is Eadie transformation of the original data

valerate (2.1 and 3.2), respectively. Perhaps, like other carbonates and thiocarbonates, the resonance stabilization of the carbonyl carbon by the alpha substituted heteroatom results in lower rates of chemical and enzymatic hydrolyses. The carbonate (3.4) was hydrolyzed faster than the thiol-carbonate (3.3)

The leaving group -XR´ significantly affected rates of substrate hydrolyses. Phenyl esters were better proteinase K substrates than the S-phenyl and S-methyl thioesters (substrates 3.8 vs 2.6, 3.7 vs 2.5, 3.6 vs 2.4, 3.5 vs 2.3, 3.4 vs 2.2, 3.2 vs 2.1, 3.2 vs 1.1). This trend was mirrored by the susceptibility of the substrates to base catalyzed nonenzymatic hydrolysis (Hammock and coworkers, unpublished data). Presumably having more stable leaving groups reduces the activation energy of the transition states for both enzymatic and nonenzymatic hydrolysis.

The new method to assay proteinase K esterase activity was tested with 29 thioesters and esters (Table 1). In addition it was examined for the determination of apparent Michaelis-Menten parameters for phenyl valerate (Fig. 1). The O-phenyl esters $CH_3(CH_2)_{n=1-2}$-S-$(CH_2)_{n=1-2}$C(O)-O-phenyl (substrates 3.6 and 3.8) and their S-phenyl thioester analogs (substrates 2.4 and 2.6) were the substrates that were hydrolyzed at the highest rates. S-phenyl thioesters were preferable for the assays since their hydrolyses can be monitored continuously, unlike the assay of phenols released from phenol esters. Microtiter plates and a 96-well plate reader permitted measuring of 96-samples at one time, resulting in a substantial saving of samples and reagents, without loss of sensitivity and accuracy (e.g., hydrolysis of substrate 2.4 can be measured at a specific activity of 5 nmol · min^{-1} · mg^{-1} at 2 : 1 ratio of signal to noise). In conclusion, the experiments demonstrate the suitability of carboxylic acid esters as surrogate substrates of O-esters of amino acids and peptides in assays of proteinase K esterase activity.

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A new pyrrolidinedithiocarbamate screening method for the determination of methylmercury and inorganic mercury relation in hair samples by HPLC-UV-PCO-CVAAS

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Abstract A new analytical screening technique for the determination of methylmercury and inorganic mercury in hair samples by HPLC-PCO-CVAAS has been developed. It is based on the extraction of mercury compounds by a buffered sodium pyrrolidinedithiocarbamate solution, separation by reversed-phase HPLC, post column oxidation by UV-irradiation, reduction with alkaline sodium borohydride, and determination by cold vapour atomic absorption detection. The standard deviation was 7% and recoveries were 90% for both compounds. The limit of detection (S/N = 3) for both compounds was calculated to be about 4 ppb.

Introduction

During the past three decades, the determination of mercury in human scalp hair has become increasingly important for monitoring environmental exposure and diagnosing diseases [1–2]. But the determination of total mercury (THg) is not a sufficient parameter for an assessment of a sample [3]. The major portion of mercury in hair is present in a complex bounded form of methylmercury (MHg) and small amounts of inorganic mercury (IHg) [4]. Deviated relations between MHg and IHg can be used for monitoring evaluating systemic intoxication and assessing nutritional status [5].

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This paper describes a new liquid extraction method for the simultaneous determination of methylmercury and inorganic mercury in human scalp hair by sodium pyrrolidinedithiocarbamate which can be injected directly into the HPLC-UV-PCO-CVAAS system [6].

Experimental

Chemicals and material

Methylmercury (by Merck) stock solution was prepared in water (HPLC grade) and mercury chloride (by Riedel-de-Haën) stock solutions were prepared in 0.1 mol/l nitric acid. The standards were prepared daily freshly by diluting the stock solutions with HPLC grade water. Sodium pyrrolidinedithiocarbamate was obtained by Fluka. All other chemicals such as acetonitrile, sodium borohydride and acetone were of analytical reagent grade. The sodium borohydride was purified from mercury by bubbling with nitrogen for 1 h before application. The standard hair material was produced ourselves by homogenizing a natural hair sample. The total mercury content of the sample was analysed 10 times with a concentration of 490 μg/kg ± 3.4%.

Apparatus

Figure 1 shows a schematic diagram of the detection system. The Thermo Separation Product Analytical HPLC system consists of a computer, pump, degasser, CVAAS detector. An RP C18 column (Hypersil-ODS, 3 μm 8 × 4.6 mm I.D.) from Grom was used for the experiments. The detection limit of the HPLC-PCO-CVAAS for methylmercury is 80 pg absolut. For the post column oxidation step a 5 m PTFE-coil from ICT with 0.3 mm I.D. was used. The PTFE-coil was irradiated by a 4 W low-pressure Hg-lamp from Philips normally used in bank-note controler. The oxidized compounds were pumped together with a nitrogen flow of 80 ml/min and a reducing solution (flow 1.5 ml/min containing 1% sodium borohydride in 0.5 mol/l NaOH) through a PTFE reducing loop with 2 m length (2 mm I.D.) into the gas-liquid-separator. The mixture runs via a glass-frit into the separator (dimensions 100 mm × 25 mm) where the reduced mercury vapour was swept by nitrogen through the moisture trap (calcium chloride) into the cold vapour atomic absorption spectrometer.

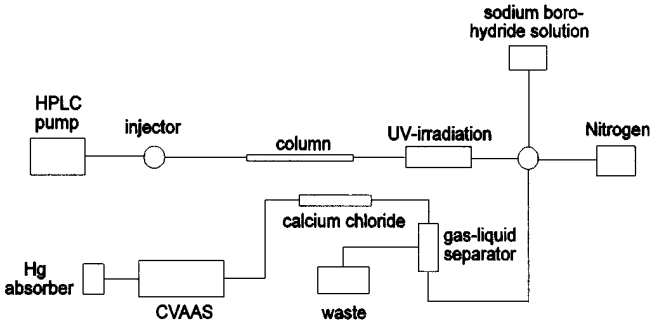

Fig. 1 Schematic diagram of the HPLC-UV-PCO-CVAAS system coupled with on-line UV-irradiation and continuous-flow mercury generation for the determination of methylmercury and inorganic mercury

Procedure

Hair samples were washed with 5 ml acetone to reduce the fat content (total mercury losses by cleaning procedure was lower than 4%). Hair samples of 200–400 mg were filled into a syringe. The hair sample was compressed with the syringe piston. 2 ml of the extraction solvent (containing acetonitrile : water, 50/50, v/v, containing 50 mmol/l sodium pyrrolidinedithiocarbamate, adjusted to pH 5.5 with ammonium acetate) was filled into the second syringe. Then the two syringes were connected by a 15 mm PTFE tube in such a way that the syringe tips were in direct contact. Then the pistons were alternatingly moved to and fro (20 times) at the beginning to soak the hair samples. This washing procedure was repeated every 5 min for 2 h. Then 50 µl of the extraction was injected into the HPLC-PCO-CVAAS-system. Eluent conditions: acetonitrile: water (65/35, v/v) with 0.5 mmol/l sodium pyrrolidinedithiocarbamate buffered at pH 6.5 with ammonium acetate. Eluent flow was 1 ml/min. Mercury was reduced by sodium borohydride (1%) adjusted to pH 14 with NaOH. Preparation of the spiked hair samples followed by adding 50 µl of a 100 µg/l methylmercury (standard solution in acetonitrile) into the 200 mg sample.

Results and discussion

The determination of methylmercury and inorganic mercury in hair samples extracted by pyrrolidinedithiocarbamate in acetonitrile/water was optimized by varying the extraction conditions. The extraction results were compared with the total mercury content. The standard deviations were 7% for both compounds. Compared to the total mercury content of the standard hair, 85% could be extracted by the new procedure. A relation between detectable CH_3Hg^+ and Hg^{2+} during the extraction procedure from standard hair is shown in Fig. 2. After an extraction time of 2 h the absorption units for the two compounds no longer increases. Figure 3 shows a chromatogram of a hair sample with high methylmercury (1) content and a second chromatogram (2) with low methylmercury content. In the first case the relation between CH_3Hg^+ and Hg^{2+} is 10 : 1.7 and in the second case 10 : 1.3. The peak heights relation of $MeHg^+/Hg^{2+}$ showed an average of 10 after a 2 h extraction time. A comparative measurement with the HCl-extraction procedure according to the Westöö'method confirmed this relation [7]. The recoveries for a spiked hair sample at the 5 ng level for methylmercury and Hg^{2+} were 90%.

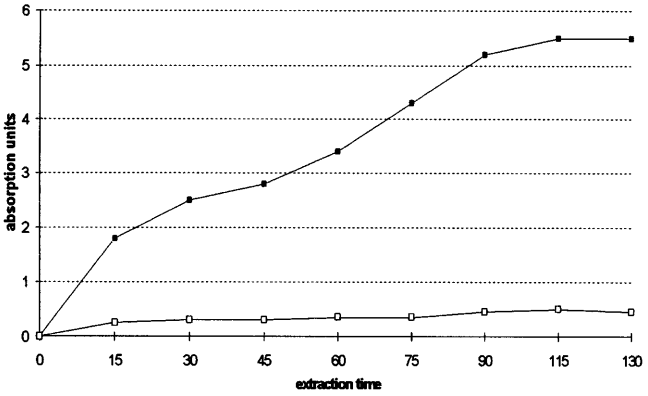

Fig. 2 Increasing of the detectable Hg^{2+} and CH_3Hg^+ during the extraction procedure. Extraction conditions: a 600 mg hair sample was extracted by 5 ml of the pyrrolidinedithiocarbamate solution, injecting 20 µl at 15 min intervals, respectively

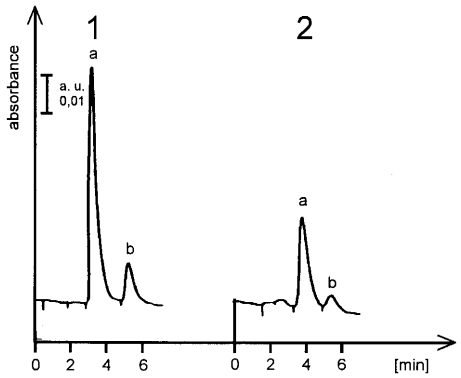

Fig. 3 *(I)* HPLC-chromatogram of a hair sample containing high methylmercury level *(a)* and low inorganic mercury peak level. *(2)* HPLC-chromatogram of a hair sample containing low methylmercury level and very low inorganic mercury

Conclusion

The determination of methylmercury and inorganic mercury in hair samples extracted by pyrrolidinedithiocarbamate in acetonitrile/water was optimized. After an extraction time of 2 h 85% of the mercury species were extracted by the pyrrolidinedithiocarbamate solution at which the $MeHg^+/Hg^{2+}$ relation could be established for diagnosing diseases. Short extraction time and simple handling are the main advantages of the new developed liquid extraction method for the simultaneous determination of methylmercury and inorganic mercury in human scalp hair.

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