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Inactivation of cathepsin D from human gastric mucosa and from stomach carcinoma by diazoacetyl-DL-norleucine methyl ester

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Enzymes from malignant tissues may be different from the corresponding enzymes of normal tissues [1, 2]. Cathepsin D from transplantable rats sarcoma has been found to differ from the rat liver enzyme in substrate and inhibitor specificity [3].

A form of cathepsin D, with high susceptibility to inhibition by pepstatin, has been found in mouse leukemia L12010 cells [4]. In this communication the effect of diazoacetyl-DL-norleucine methyl ester on cathepsin D from human stomach mucosa and stomach carcinoma is described. Cathepsin D from stomach carcinoma was found to be more susceptible to inactivation by the diazoketone than the enzyme from normal gastric mucosa. This effect was found to hold in the homogenate, as well as in the partially purified enzyme.

Samples of gastric mucosa (20–35 g) were obtained from resected stomachs of patients undergoing gastrectomy for treatment of duodenal ulcer. Tumour samples were obtained from patients undergoing treatment for stomach carcinoma. All tumours utilized were well differentiated adenocarcinomas. Tissue samples were homogenized within a few hours after gastrectomy or were kept at 4° for a period of 1–2 days before homogenization.

Bovine haemoglobin Type II, Sepharose 4B-200, ovalbumin, chymotrypsinogen, cytochrome c and soya-bean trypsin inhibitor were purchased from Sigma Chemical Co. Sephadex G-75 was obtained from Pharmacia (Uppsala). Diazoacetyl-DL-norleucine methyl ester was a product of Nutritional Biochemicals Corporation. Cyanogen bromide was purchased from Matheson, Coleman & Bell. Carbowax 20-M (flakes) was obtained from Mann Research Laboratories.

Acrylamide and bisacrylamide were obtained from BIO-RAD Laboratories. Ammonium persulfate and Amidoschwartz-10B were purchased from Merck.

Assay of cathepsin D activity was as described previously [5]. Inactivation studies of cathepsin D by the diazoketone were carried out as follows: Preincubation mixtures (in a final volume of 0.5 ml) of cathepsin D and diazoacetyl-DL-norleucine methyl ester consisted of cathepsin D preparation (corresponding to 0.30–0.66 μmoles of tyrosine produced per hr at 37° and pH 3.5), diazoacetyl-DL-norleucine methyl ester as shown in Fig. 1, 0.1 M-sodium acetate buffer, pH 5.0, 0.5 mM-$CuSO_4 \cdot 2H_2O$ and 10% (v/v) ethanol. Diazoacetyl-DL-norleucine methyl ester was dissolved in ethanol immediately before the start of the experiment. After incubation for 30 min [6] at 37°, 5.0 ml of haemoglobin solution [5] was added for the determination of cathepsin D activity [5]. The haemoglobin–agarose resin was prepared according to the method of Porath *et al.* [7]. Haemoglobin was coupled to agarose at pH 7.0 for 24 hr. Protein was determined by the microbiuret method [8]. Disc electrophoresis at pH 8.75, in gels containing 8% acrylamide, of partially purified enzyme from normal gastric mucosa and from stomach carcinoma was performed. The gels were stained by being placed in a 0.5% solution of Amidoschwarz-10B in 20 methanol-16 water-4 glacial acetic acid and they were destained by washing with 7% (v/v) acetic acid solution. Samples for electrophoresis were prepared by using portions of the purified enzyme preparations (30–60 μg) to which sucrose to a final concentration of 20% (w/v) was added. To determine which electrophoretic band contained active enzyme, gels were run and afterwards were sliced transversely into

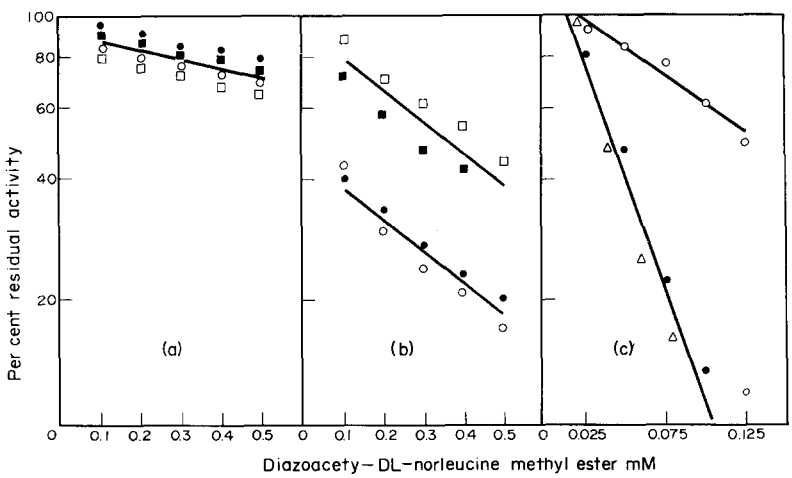

Fig. 1. Inactivation of cathepsin D by diazoacetyl-DL-norleucine methyl ester. (a) Stomach mucosa homogenates, and (b) stomach carcinoma homogenates from four different individuals in each instance. (c) Partially purified preparation of cathepsin D from stomach mucosa (○), and stomach carcinoma (●, △). For details see text.

3 mm sections. The gel slices were incubated in 0.5 ml of water at 4° overnight and the extracts were used for enzymatic assay.

Cathepsin D was purified by a modification of the method of Smith and Turk [9]. All operations were carried out at 4°, unless otherwise specified. Tissue samples were homogenized in a Sorvall (Omni Mixer 17220) homogenizer, for a period of 3 min, and in a ratio of 2.5 ml of water per gram of tissue. The temperature of the material was maintained close to 4° during homogenization, by immersing the container in an ice–ethanol bath. TRITON X-100 (0.2% w/v) was then added, and the preparation was stirred for 1 hr. The preparation was centrifuged at 16,000 *g* for 10 min. The supernatant was used for enzyme studies or for purification.

The preparation was made 50 mM with respect to Tris–HCl pH 8.0, and acetone was added by drops while stirring at the same time, while the temperature of the preparation was being lowered to −15°. When the acetone added was 33 per cent of the total volume, the preparation was stirred for 5 min, was allowed to stand at −20° for 10 min, and finally centrifuged at −20° and at 16,000 *g* for 10 min. Acetone was added to the supernatant to 66 per cent of the total volume, and the preparation was centrifuged as previously. The precipitate was taken up with water. The resuspended precipitate was placed in a dialysis bag and surrounded with Carbowax flakes until it was reduced to half its original volume. The preparation was dialysed overnight *v.* water, and was centrifuged at 16,000 *g* for 10 min. The pH of the supernatant was brought to 3.8 with 1 M sodium acetate buffer pH 3.5, and the precipitate was centrifuged at 16,000 *g* for 10 min. The supernatant (20–30 ml), containing 3–12 mg protein per ml, was placed on an agarose column which had been coupled to haemoglobin as described by Smith and Turk [9]. The column had been equilibrated with a 10 mM pH 4.1 sodium acetate buffer. The rest of the procedure was as described by Smith and Turk, with the exception of Sephadex G-75 having been used in place of Sephadex G-100. No cathepsin D activity was found in the column wash of the agarose–haemoglobin column. Cathepsin D activity was obtained from the Sephadex column in a single peak. The overall purification effected was 66-fold. A summary of these results is given in Table 1. Closely similar results were obtained with the enzyme from gastric mucosa. Cathepsin D from human gastric mucosa has been purified, along different lines, by Mangla *et al.* [10].

After Sephadex G-75 chromatography the partially purified preparation of cathepsin D from gastric mucosa and stomach carcinoma showed two stainable bands in electrophoresis in polyacrylamide gel. In each case only one of the two bands exhibited cathepsin D activity on assay after elution. The electrophoretic mobility of the cathepsin D containing band of gastric mucosa, was lower than the mobility of the band from stomach carcinoma.

The heat sensitivity of cathepsin D was studied with the partially purified enzyme from normal gastric mucosa, and from stomach carcinoma. Samples containing 0.05 M Tris–HCl pH 8.0, and cathepsin D were placed in a water bath at 60°. Samples were removed every 3 min up to a total period of 15 min, and immediately immersed in an ice-alcohol bath. Portions were assayed for cathepsin D activity. Inactivation of cathepsin D was the same for the enzyme from normal gastric mucosa and stomach carcinoma, with a first order rate constant of 1.74/hr in both cases.

The Sephadex C-75 column used for the purification of the enzyme was calibrated by running ovalbumin, chymotrypsinogen, cytochrome *c*, and soya-bean trypsin inhibitor through the column. The molecular weight of cathepsin D was calculated from a log molecular weight *v.* effluent volume/void volume plot, and was found to be 63,800, both for the enzyme from normal gastric mucosa and that from stomach carcinoma. A comparison of the molecular weight determinations of cathepsin D from various sources is given by Ferguson *et al.* [11].

The results of the inactivation experiments with homogenates from normal stomach mucosa, and from homogenates from stomach carcinoma are shown in Fig. 1. Results are plotted according to equation (6) of Rakitzis [5]. It will be seen that a straight line relationship exists between the log per cent residual activity and the initial inhibitor concentration $[I]_0$. However, the lines drawn through the points meet the log per cent residual activity axis below the origin of the graph. These findings are compatible with the existence of two forms of cathepsin D, one of which is fully inactivated at low inhibitor concentrations. The modification of equation (6) of Rakitzis [5] to describe the inactivation of the mixture of two enzyme forms is:

$$(v_i/v) = c_a e^{-(k_a/K_{ia}c)[I]_0} + c_b e^{-(k_b/K_{ib}c)[I]_0} \qquad (1)$$

where c_a and c_b are the fractional concentrations of the two enzyme forms in the preparation, the rest of the notation being as in equation (6) of [5].

It will be observed from Fig. 1 that the slopes of all four inactivation experiments with homogenates from stomach carcinoma are the same, while the intercepts on the per cent residual activity axis are different for each experiment. This suggests that the highly sensitive form of cathepsin D is present in different proportions in each case. Results with homogenates from stomach mucosa are much more uniform (Fig. 1).

The value for $-k/K_i c$ for cathepsin D in homogenates from stomach mucosa is 0.50 mM^{-1}, while that in homogenates from stomach carcinoma is 1.78 mM^{-1}. The corresponding values for the partially purified enzyme are 5.15 mM^{-1} for the preparation from stomach mucosa, and 22.1 mM^{-1} for the preparation from stomach carcinoma. These findings indicate that cathepsin D from stomach carcinoma is some three times more susceptible to inactivation by diazoacetyl-DL-norleucine methyl ester than the enzyme from stomach mucosa, whether the preparation is in the partially purified form or at the homogenate stage.

Table 1. Purification of cathepsin D from stomach carcinoma

Fraction	Total vol. (ml)	Total protein (mg)	Total activity*	Specific activity†	% yield
Crude homogenate	70	7967	3920	0.46	100
Centrifuged homogenate	65	1982	3640	1.84	92.8
Acetone 33–66%	52	1040	1968	1.89	50.2
Acetate	42	160	490	3.06	12.5
Agarose-Hb eluate	37	14.8	271	18.2	6.91
Sephadex G-75 eluate	40	3	94.7	31.5	2.41

* In μmoles tyrosine produced per hr at 37° and at pH 3.5.
† In μmoles tyrosine produced per hr/mg protein.

It is not clear whether the higher susceptibility of cathepsin D when in the purified form, is due to removal of the homogenate protein bulk, or whether the isoenzyme which is less sensitive to the diazoketone has been entirely lost during the purification procedure.

Diazoketones have been shown to inactivate acid proteinases from a variety of sources [6, 11–17]. The esterification of an aspartyl residue in pepsin by 1-diazo-4-phenylbutanone-2 has been reported [18], and the primary structure of the amino acid sequence containing the reactive residue has been determined [18–21]. The higher susceptibility of cathepsin D from stomach carcinoma, in comparison to the susceptibility of this enzyme from gastric mucosa, indicates the presence of a different form of the enzyme in the tumour tissue.

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An improved dual-wavelength spectrophotometric assay for dopamine-β-hydroxylase

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We report an improved dual-wavelength spectrophotometric assay for dopamine-β-hydroxylase (DBH) which has higher sensitivity and reproducibility than our previously reported method and can be applicable to any crude tissues of guinea pigs and rats to measure the maximum velocity at saturated concentrations of substrate and cofactor under the optimum conditions and complete inactivation of endogenous inhibitors [1].

Tyramine hydrochloride was obtained from Merck; catalase (crystal suspension) from Boehringer; *N*-ethylmaleimide and octopamine hydrochloride from Sigma; Cutscum (isooctylphenoxypolyethoxyethanol-containing detergent) from Fisher Scientific Co., Dowex-50W-X4 from Dow Chemical Co. Pargyline hydrochloride and fusaric acid were kindly provided from Abbott Laboratories and Banyu Pharmaceutical Co., respectively. Homogeneous DBH was prepared from bovine adrenal medulla by the method of Foldes *et al.* [2]. Superoxide dismutase purified from bovine erythrocytes by the method of McCord and Fridovich [3] was kindly provided by Dr. Miki Akino (Tokyo Metropolitan University, Tokyo). Tissues of guinea pigs or rats were homogenized and the enzyme was solubilized, as reported previously [1].

The improved Standard Assay System for DBH (total volume, 1.0 ml, the final concentration of each reagent in the parentheses) was prepared as follows. The enzyme solution was added to a 15 ml centrifuge tube. Water was added to make up 500 μl of enzyme preparation. Fifty μl of 2 mM fusaric acid (100 μM) were included in another enzyme preparation for the blank. Twenty μl of an internal standard solution containing 2.00 nmoles of octopamine were added to another blank incubation mixture. Then 100 μl of 2 M sodium acetate buffer, pH 5.0 (0.2 M), 150 μl of 0.2 M *N*-ethylmaleimide (30 mM), 50 μl of 200 μM $CuSO_4$ (10 μM), 25 μl of aqueous solution (20 mg/ml) of catalase (25,000 U, 500 μg), and 25 μl of 40 mM pargyline hydrochloride (1 mM) were added, and the solution was mixed to inactivate the endogenous inhibitors and monoamine oxidase in the enzyme preparation. Then the following reaction mixture was added: 50 μl of 0.2 M ascorbic acid (10 mM), 50 μl of 0.2 M sodium fumarate (10 mM), and 50 μl of 0.4 M tyramine hydrochloride (20 mM). The reaction mixtures were incubated at 37° for 45 min in air with continual shaking. The incubation was stopped by adding 0.2 ml of 3 M trichloroacetic acid in an ice bath and the mixture was immediately centrifuged at 2500 rpm for 10 min. The supernatant fluid was immediately transferred to a small glass column (0.5 × 10 cm) of Dowex-50W-X4 (H^+, 200–400 mesh, packed volume, 0.2 ml). The tube and the precipitate were washed with 1 ml of water,