The inhibition of cathepsin B by plasma haptoglobin biochemistry (enzymes, metabolism)[1]

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Summary. Purified haptoglobin partially inhibits the activity of a lysosomal thiol proteinase, cathepsin B. This inhibition is reversible in the presence of monospecific antiserum to haptoglobin.

The lysosomal endopeptidase cathepsin B plays a major role in the normal catabolism of proteins[2]. In tissue injury or cell death cathepsin B and other lysosomal proteases may be exposed to extracellular substrates. It has been suggested that proteolysis of these substrates may lead to the release of pharmacological mediators of inflammation[3,4] and hence play an important role in the inflammatory process.

Serum haptoglobin concentrations are increased in conditions associated with inflammation and it has been suggested that haptoglobin is an inhibitor of cathepsin B[5]. This suggestion has been disputed[6]. This controversy is partly related to the difficulty in obtaining a purified preparation of haptoglobin. In the preparation of haptoglobin by column chromatography the haptoglobin fraction is often contaminated with alpha-2-macroglobulin (α_2M) a known inhibitor of cathepsin B. In this study an attempt was made to exclude the effects of α_2M and to see if purified haptoglobin inhibited the activity of the enzyme.

Materials and methods. Human liver cathepsin B, α-N-benzoyl-Arg-Arg-2-naphthylamide, a synthetic substrate for cathepsin B and Fast Garnet were gifts from Dr A. Barrett of Strangeways Laboratory, Cambridge. Antisera to human plasma proteins were obtained from the Immunodiagnostic Research Laboratories, Birmingham, U.K. and Dako-Immunoglobulins Ltd, Copenhagen, Denmark. Cyanogen Bromide Activated Sepharose 4B was purchased from Pharmacia (G.B.) Ltd, London, U.K. Diaflo Ultrafiltration Membranes were obtained from the Amicon Corporation, Lexington, Massachusetts, USA. Human immunoglobulin G was a gift of the Department of Immunology, Addenbrooke's Hospital, Cambridge.

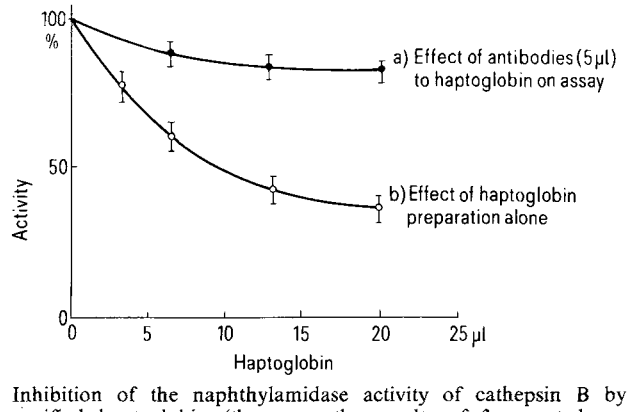
a) Characterization of cathepsin B. The purity of the preparation was checked by zone electrophoresis in 1% agarose made up in 0.05 M phosphate buffer pH 7.4. The activity of the enzyme was determined by a method previously described[7]. An aliquot containing 200 µg/ml of protein was prepared in 0.05 M acetate buffer pH 5.5 for use in the enzyme assays, 10 µl (2 µg) was used per assay tube.

b) Purification of haptoglobin. Saturated ammonium sulphate was added dropwise to 10 ml human plasma, the final salt saturation being 33% (253 g/l). The globulins were precipitated and the supernatant was discarded. The precipitate was redissolved in 0.05 M phosphate buffer pH 7.4. This solution was passed down a Sephadex G200 column (100 cm long × 1.6 cm diameter). The haptoglobin fraction was identified by testing against antibodies to haptoglobin using radial immunodiffusion[8]. This fraction also contained α_2M and IgG. Monospecific antibodies to these contaminating proteins were coupled to Cyanogen Bromide Activated Sepharose 4B as recommended by the manufacturer. The haptoglobin fraction was then allowed to equilibrate with each solid phase immunoadsorbent and the fraction was re-tested by 2 dimensional immunoelectrophoresis[9] to ensure that the contaminating proteins had been removed completely. This fraction was concentrated using Amicon Diaflo Ultrafilters with a 20,000 mol. wt cut off. The purity of the haptoglobin preparation was determined by adding haemoglobin to a sample and subjecting the mixture to polyacrylamide gel electrophoresis using a discontinuous buffer of pH 8.6. The haptoglobin haemoglobin bands were identified by staining for peroxidase activity[10]. Gels were run simultaneously in which the protein bands were fixed in 10% trichloroacetic acid and stained with Coomassie Blue. Immediately preceding the assay the pH of the purified haptoglobin fraction was adjusted to 6.0, optimal for cathepsin B activity, and the molarity to 0.1 using 0.25 M phosphate buffer. The final concentration of haptoglobin (3.0 mg/ml) was determined by the method of Lowry et al.[11]. Aliquots of this fraction were then added to the cathepsin B preparation and the enzyme was assayed in the presence and absence of haptoglobin.

1 ml (10 mg) of rabbit anti-human haptoglobin serum was passed down a Sephadex G100 column (25 cm long, 1.6 cm diameter) to remove the azide which inhibits the enzyme. Aliquots of this fraction 5 µl (25 µg) were pre-incubated for 4 h at 4 °C with 15 µl (45 µg) haptoglobin preparation and used to test for reversal of inhibition on 10 µl (2 µl) of cathepsin B.

Results and discussion. The purified preparation of cathepsin B (200 µg/ml) on agarose electrophoresis demonstrated 2 bands. The major band had α_1 mobility. This result agrees with the reported acidic nature of cathepsin B which has an isoelectric point 4.5–5.5[12]. The 2nd minor band was an albumin contaminant as shown by radial immunodiffusion (not shown).

On polyacrylamide gel electrophoresis haptoglobin haemoglobin complexes usually demonstrate multiple bands



Inhibition of the naphthylamidase activity of cathepsin B by purified haptoglobin (these are the results of 3 repeated experiments). Activity in the presence of haptoglobin expressed as a percentage of the activity of the enzyme in its absence. The bars represent the 95% confidence limits. There was no further inhibition with larger haptoglobin values (> 15 µl) which corresponds to an approximate molar ratio for haptoglobin:cathepsin B of 4:1. Curve A represents the enzyme activity when the antiserum to haptoglobin is pre-equilibrated with purified haptoglobin. Human IgG (control) in similar protein concentrations to the haptoglobin antiserum (2.5 mg/ml) demonstrated a mean of 85% of the original activity which is similar to that obtained with bovine serum albumin. Amounts greater than 50 µl (125 µg) did not reduce activity significantly below 85%. Lesser amounts demonstrated less inhibition and larger amounts did not reduce the activity to less than a mean of 85%.

when stained for peroxidase activity. There were 2 extra faint bands on the corresponding gel stained with Coomassie Blue which did not have peroxidase activity (not shown). Their approximate mol. wts were 100,000 and 150,000. However, 2-dimensional immunoelectrophoresis of the purified haptoglobin fraction with rabbit anti whole human serum antibodies demonstrated a single peak. A similar result was obtained with rabbit anti human haptoglobin antibodies. Although it is uncertain whether these 2 bands are protein contaminants they appear to co-precipitate with haptoglobin on immunoelectrophoresis. The purified haptoglobin preparation did not cross react with antiserum to α_2M, IgG, IgA or IgM the only other proteins which have been suggested to have some inhibitory action against cathepsin B. The immunoglobulins are only inhibitory in very high concentration[6].

The purified fraction inhibited the activity of cathepsin B and this effect could be reversed by the use of monospecific antiserum to haptoglobin up to a maximum mean of 85% of its original activity. The inhibition was partial as in the presence of excess haptoglobin approximately 40% of the activity was still retained. The reversal of inhibition by antibodies to haptoglobin may be due to the greater affinity of the antibodies for haptoglobin.

This effect is clearly not due to α_2M and suggests that haptoglobin is an inhibitor of cathepsin B and it remains to be determined if it is associated with the general thiol proteinase inhibitor isolated from human plasma[13,14].

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2 K. Otto, in: Tissue Proteinases, p. 1. Ed. A.J. Barrett and J.T. Dingle. North Holland, Amsterdam 1971.
3 A. Janoff, A. Rev. Med. *23*, 177 (1972).
4 L.M. Greenbaum, in: Proteases and Biological Control, p. 223. Ed. E. Reich, D.B. Rifkin and E. Shaw. Cold Spring Harbor Laboratory, New York 1975.
5 O. Snellman and B. Sylven, Nature *216*, 1033 (1976).
6 P.M. Starkey and A.J. Barrett, Biochem. J. *131*, 823 (1973).
7 A.J. Barrett, Analyt. Biochem. *47*, 280 (1972).
8 G. Mancini, A.O. Carbonara and J.F. Heremans, Immunochemistry *2*, 235 (1965).
9 H.G.M. Clarke and T. Freeman, Clin. Sci. *35*, 403 (1968).
10 J.V. Pastewka, R.A. Reed, A.T. Ness and A.C. Peacock, Analyt. Biochem. *51*, 152 (1973).
11 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
12 A.J. Barrett, Biochem. J. *131*, 809 (1973).
13 M. Sasaki, K. Minakata, H. Yamamoto, M. Niwa, T. Kato and N. Ito, Biochem. biophys. Res. Commun. *76*, 917 (1977).
14 H.C. Ryley, Biochem. biophys. Res. Commun. *89*, 871 (1979).

Effect of spike disease on the 5′- and 3′-nucleotidase activities in sandal plants

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Summary. In the sandal plant affected by spike disease, caused by mycoplasma-like organisms, the changes occurring in the 5′- and 3′-nucleotidase activities were studied and their possible significance discussed.

Study of 5′-nucleotidase activity has received attention in virus-infected animal tissues[1,2]. Information on changes occurring in the activity of this enzyme in infected plant tissue is scanty. In the sandal plant (*Santalum album* L.) affected by spike disease, caused by mycoplasma-like organisms, a disturbance in the nucleic acid metabolism has been reported[3]. It was therefore of interest to examine the changes occurring in the 5′-nucleotidase (5′-Ntdase) and 3′-nucleotidase (3′-Ntdase) activities in the spiked sandal plant.

Materials and methods. Leaf samples were taken from 6 healthy and 6 spiked sandal plants as described earlier[4]. Preparation of the enzyme extract and assay of the nucleotidase activity were done adopting the method described by Yee Foong Lai et al.[5]. The leaves (2 g) were homogenized with pre-cooled $NaHCO_3$ buffer (0.05 M, pH 7.4), and the homogenate filtered through a cloth, made up to 25 ml with the same buffer and centrifuged (3500 rpm, 10 min). The supernatant solution formed the enzyme extract. The reaction mixture (2 ml), among the other components referred to in the procedure described by Yee Foong Lai et al.[5], contained 0.5 ml enzyme extract and 2.5 mM nucleotide substrate (5′- or 3′-monophosphate of adenosine/guanosine/cytidine/uridine as needed) and was incubated for 2 h at 37 °C. In the control, addition of trichloroacetic acid preceded the addition of the enzyme extract. The liberated Pi was estimated in an aliquot of the reaction mixture. The enzyme activity was expressed as µg Pi liberated/40 mg tissue/2 h under the conditions of the experiment.

Results and discussion. Results obtained are presented in the table. From the table it can be seen that, during infection by spike disease, characteristic changes occurred in the 5′- and 3′-Ntdase activities. Compared to the healthy, in the diseased plant the 5′-Ntdase activity in respect of 5′-GMP, 5′-CMP and 5′-UMP increased in the young leaves and decreased in the mature ones; similar variations occurred in the enzyme activity in respect of 5′-AMP also, but to a less extent. While the activity of 5′-Ntdase increased from young to mature stage of the leaves in the healthy plant, it was the opposite in the spiked one. In contrast, the 3′-Ntdase activity declined from the young to the mature stage of the leaves in both healthy and spiked plants, the activity in the latter tending to remain at a relatively higher level throughout, more so in respect of 3′-AMP.

It has been suggested that 5′-Ntdase regulates the level of ribonucleotides for ribonucleic acid (RNA) synthesis in the tissue[6,7]. However, 5′-Ntdase activity in sandal did not show a determinant effect on the actual RNA level in the tissue, since despite the opposite trends of variations observed in the 5′-Ntdase activity in the healthy and spiked plants, the RNA level in both the plants was at a maximum level in the young leaves[3]. Nevertheless, it is possible that the increased activity of 5′-Ntdase in the young leaves of the diseased plant, where it has been indicated that multiplication of the