# DETERMINATION OF ESTERASE ACTIVITY OF HUMAN AND ANIMAL SERINE PROTEINASES, USING FLUOROGENIC AMINO-ACID ESTERS AS SUBSTRATE

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Activation and inactivation of proteolysis in the blood and tissues is one of the mechanisms for the maintenance of homeostasis in man and animals. Secretion of serine proteinases by activated neutrophils and the diversion of pancreatic enzymes into the blood stream in pathological states lead to raised levels of activity of these enzymes and intensification of their inactivation by the system of inhibitors.

The principal serine proteinases secreted by neutrophils are elastase and cathepsin G [7]. As substrate for determination of activity of these proteinases, semispecific synthetic esters of amino acids and peptides are frequently used. To determine activity of chymotrypsin and cathepsin G, the *p*-nitrophenyl ester of *tert*-butyloxycarbonyl-L-tyrosine [3], and the ethyl ester of N-benzoyl-L-tyrosine [8] are used, whereas to determine the esterase activity of elastase, the *p*-nitrophenyl ester of *tert*-butyloxycarbonyl-L-alanine is widely used [10].

In some cases, for example, in the study of the direct esterase activity of human and animal blood serum, the sensitivity of spectrophotometric methods is inadequate. We have therefore used two synthetic fluorogenic substrates, namely the 4-methylumbelliferyl esters of N-benzyloxycarbonylphenylalanine (Z-Phe-OMC) and *tert*-butyloxycarbonyl-L-alanine (BOC-Ala-OMC). As fluorogenic label in the substrates we used 4-methylumbelliferone – a fluorescent indicator widely used for the synthesis of low-molecular-weight substrates for glycosidases, lipases, and so on [4]. According to data in the literature [5] the 4-methylumbelliferyl ester of N-benzyloxycarbonylphenylalanine can be used as substrate for determination of $\alpha$-chymotrypsin activity ("Boehringer," Germany), when 2 ng of protein is sufficient to allow activity of the enzyme to be determined.

In the present investigation the above ester (substrate I) was used to determine the esterase activity of chymotrypsin from the bovine pancreas, cathepsin G from human, rat, and hog neutrophils, and the chymotrypsinlike activity of human blood serum, rat blood serum (plasma), and hog blood plasma, whereas the 4-methylumbelliferyl ester of *tert*-butyloxycarbonyl-L-alanine (substrate II) was used to determine elastase activity from human, rat, and hog neutrophils, and esterase activity in human blood serum, hog blood plasma, and rat blood plasma.

## EXPERIMENTAL METHOD

Cathepsin G and elastase from human and animal neutrophils were obtained by the method described previously [1]. Home-produced (Olaine) chymotrypsin from bovine pancreas was used in the experiments. Z-Phe-OMC (substrate I) and BOC-Ala-OMC (substrate II) were obtained by the method described previously [2].

Esterase activity of the human and animal blood serum enzymes was measured in cuvettes of a "Perkin Elmer 3000" spectrofluorometer.

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TABLE 1. Chymotrypsin-Like Activity of Human and Animal Enzymes and Blood Serum (Plasma) Determined with the Aid of Substrate I (M ± m)

| Enzyme or blood serum (plasma) | Chymotrypsin from bovine pancreas |
|---|---|
| Chymotrypsin from bovine pancreas | 5.35 mmoles/min·mg protein |
| Cathepsin G from human neutrophils | 2.0 mmoles/min·mg protein |
| Cathepsin G from rat neutrophils | 1.4 mmoles/min·mg protein |
| Cathepsin G from hog neutrophils | 1.1 mmoles/min·mg protein |
| Human blood serum | 136 ± 6 mmoles/min·liter (n = 43) |
| Rat blood serum | 1240 ± 103 mmoles/min·liter (n = 6) |
| Rat blood plasma | 1920 ± 175 mmoles/min·liter (n = 5) |
| Hog blood plasma | 75 ± 9 mmoles/min·liter (n = 3) |

TABLE 2. Elastase-Like Esterase Activity of Human and Animal Enzymes and Blood Serum (Plasma), Determined with the Aid of Substrate II (M ± m)

| Enzyme or blood serum (plasma) | Activity of hydrolysis of substrate II |
|---|---|
| Elastase from human neutrophils | 0.3 mmoles/min·mg protein |
| Elastase from hog neutrophils | 0.26 mmoles/min·mg protein |
| Elastase from rat neutrophils | 0.26 mmoles/min·mg protein |
| Human blood serum | 98 ± 3 mmoles/min·mg protein (n = 43) |
| Rat blood plasma | 450 ± 52 mmoles/min·liter (n = 11) |
| Hog blood plasma | 85 ± 12 mmoles/min·liter (n = 3) |

Enzyme activity was measured at an excitation wavelength of 360 nm and emission wavelength of 455 nm, in 0.01 M sodium-phosphate buffer, pH 7.4, at 20 ± 1°C, and with substrates in a concentration of $(1\text{-}3)\cdot10^{-5}$ M for 2 min.

To determine activity of the pure enzymes, from 2 to 0.1 $\mu$g of enzyme was introduced into the fluorometer cuvette, whereas to test the blood serum esterase activity 5 $\mu$l of serum was added. Rat blood serum (plasma), for determination of esterase activity based on hydrolysis of substrate I, was diluted 1:10 with physiological saline.

The substrates were dissolved in dimethylsulfoxide and the level of spontaneous hydrolysis of the substrates was measured by adding 10 $\mu$g of the original substrate solution to the cuvette containing phosphate buffer (3 ml). The level of spontaneous hydrolysis was 60 ± 20, on a scale of measurement of 1000 conventional units (sensitivity of the instrument used was 5). Enzymes or human and animal blood serum (plasma) were applied from a microsyringe to the wall of the cuvette containing phosphate buffer, and 10 $\mu$l of the original solution of substrate was applied, also from a microsyringe, to the opposite wall of the cuvette; the cuvettes were then covered and the contents mixed by shaking for 10 sec. Activity was measured, taking readings of the instruments after 30 sec and 1 and 2 min.

A calibration curve was plotted for 4-methylumbelliferone. Protein was measured by Lowry's method.

## EXPERIMENTAL RESULTS

Table 1 gives values of specific esterase activity of chymotrypsin and cathepsin G from human and animal neutrophils, and activity of human and animal blood serum (plasma), obtained with the aid of substrate I.

Table 2 gives values of elastaselike esterase activity of elastase from human, hog, and rat neutrophils, human blood serum, and hog and rat blood serum (plasma), obtained with the aid of substrate II.

It can be concluded from analysis of these results that there are significant differences in the levels of both chymotrypsinlike and elastaselike esterase activity of human and animal blood serum (plasma). These differences were most marked for rat blood serum (plasma) compared with human blood serum and hog blood plasma. Activity of the purified enzymes (elastase and cathepsin G) from human neutrophils, on the other hand, did not differ significantly from activity of these enzymes from hog and rat neutrophils, in good agreement with data in the literature [1, 9].

Direct esterase activity of human and animal blood serum, determined with respect to hydrolysis of fluorogenic substrates I and II was evidently attributable to serine proteinases (elastase and chymotrypsinlike enzymes), present in the form of a complex with $\alpha_2$-macroglobulin – a polyvalent high-molecular-weight inhibitor of blood serum proteinases. This inhibitor inactivated 86% of chymotrypsinlike enzymes and 10% of elastase, on entering the blood stream. The proteolytic activity of proteinases in the form of a complex with $\alpha_2$-macroglobulin is lost, but they preserve their ability to hydrolyze low-molecular-weight substrates [6]. Our suggested method of determining direct elastaselike and chymotrypsinlike activity enables both esterase activity of highly purified elastase and cathepsin G from human and animal neutrophils and blood serum (plasma) esterase activity to be determined.

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