AMINOPEPTIDASES OF RAT TESTIS

III. ACTIVITY OF DIPEPTIDYL AMINOPEPTIDASES I AND II IN NORMAL AND EXPERIMENTAL CONDITIONS

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Summary. The lysosomal enzymes, dipeptidyl Aminopeptidase I and II, were found in rat testicular tissue. Enzyme I hydrolysed seryl-tyrosyl- β -naphthylamide (Ser-Tyr- β -NA) optimally at pH 4.5 and was activated by thiol-groups and chloride ions. Enzyme II had a model substrate of Lys-Ala- β -NA, which was optimally hydrolysed at pH 5.5. These enzymes were partially separated by DEAE-cellulose chromatography.

The major activity of Enzyme I appeared to be in the interstitial tissue, since isolated seminiferous tubules contained only trace amounts of this activity. By contrast, Enzyme II appeared to be present in both the interstitial and tubular tissues.

During the pubertal maturation of the testis, a gradual increase in Enzyme I activity was observed. Cryptorchidism caused an initial suppression in 5 days, followed by a rapid increase. These findings indicate a functional correlation of Enzyme I activity with the Leydig cells.

Enzyme II activity showed only a slight decrease during puberty. After cryptorchidism, an initial increase was encountered at 5 to 8 days, followed by a rapid decline. These observations further indicate the presence of this enzyme in both the interstitial and tubular tissues.

Cadmium chloride treatment caused an initial increase of Enzyme I activity which was coincidental with the rapid destruction of the seminiferous epithelium. Subsequently, a decline followed which was concurrent with the destruction of the interstitial cells. Enzyme II showed a more rapid suppression soon after cadmium chloride treatment.

INTRODUCTION

Most intracellular aminopeptidases hydrolyse a single amino acid at a time from the NH_2 -terminal end of a protein or a peptide, but a variety of aminopeptidases, which hydrolyse two amino acids at a time from the NH_2 -terminus, have been characterized. At least four such dipeptidyl aminopeptidases are known to occur in mammalian tissues.

Dipeptidyl Aminopeptidase I is a lysosomal enzyme with optimum activity at pH 4.0 to 4.5 and is also able to hydrolyse many synthetic substrates, which can be conveniently utilized for quantification (McDonald, Ellis & Reilly, 1966; McDonald, Zeitman, Reilly & Ellis, 1969; McDonald, Callahan, Ellis & Smith, 1971). Seryl-tyrosyl- β -naphthylamide (Ser-Tyr- β -NA) is the model substrate for this enzyme, which has also been identified as cathepsin C (McDonald *et al.*, 1969).

Dipeptidyl Aminopeptidase II is also a lysosomal enzyme with pH-optimum at 5.5 and has a substrate specificity markedly different from that of Enzyme I (McDonald, Leibach, Grindeland & Ellis, 1968; McDonald, Reilly, Zeitman & Ellis, 1968; McDonald *et al.*, 1971). Lysyl-alanyl- β -naphthylamide (Lys-Ala- β -NA) has been used as substrate for its quantification (Vanha-Perttula, 1969).

Dipeptidyl Aminopeptidase III is a soluble enzyme with an optimum at pH 8 to 9 (Ellis & Nuenke, 1967; Hopsu-Havu, Jansén & Järvinen, 1970). It can be quantified with arginyl-arginyl- β -naphthylamide (Arg-Arg- β -NA) or Leu-Ala- β -NA as substrate. Dipeptidyl Aminopeptidase IV has been localized in the microsomal fraction and has an optimum at pH 7.8 (Hopsu-Havu & Sarimo, 1967). Glycyl-prolyl- β -naphthylamide (Gly-Pro- β -NA) has been used as a synthetic substrate for its quantification.

In the present investigation, the lysosomal dipeptidyl Aminopeptidases I and II were studied in rat testicular tissue using DEAE-cellulose chromatography. The isolated enzymes were characterized and their activity determined during the pubertal maturation of the testis, after cryptorchidism as well as after cadmium chloride treatment. The findings were compared to those obtained for other lysosomal enzyme activities reported previously (Vanha-Perttula & Nikkanen, 1972).

MATERIALS AND METHODS

Tissue preparation

Testicular tissue obtained from adult Sprague-Dawley albino rats was prepared for subcellular fractionation and sucrose density gradient centrifugation, as well as for DEAE-cellulose chromatography, by the methods already defined (Vanha-Perttula, 1973a). The procedures for centrifugation and chromatography have also been previously described (Vanha-Perttula & Nikkanen, 1972).

Measurement of enzymatic activity

The hydrolysis of Ser-Tyr- β -NA (Cyclo Chem. Corp., Los Angeles, Calif.) in the quantification of Enzyme I activity and that of Lys-Ala- β -NA (Fox Chem. Co., Los Angeles, Calif.) for Enzyme II was carried out by the method of Takenaka (1964). The incubation medium for Enzyme I consisted of 0.5 ml buffer solution (succinic acid 140 mmol/litre, 2-mercaptoethanol 10 mmol/litre, NaCl 10 mmol/litre adjusted to pH 4 with sodium hydroxide), 0.25 ml enzyme solution and 0.25 ml substrate solution (1 mmol/litre). The incubation medium for Enzyme II activity contained 0.5 ml buffer (succinic acid 20 mmol/

litre and NaOH to adjust to pH 5.5), 0.25 ml enzyme solution and 0.25 ml substrate solution (1 mmol/litre).

The protein content of the enzyme samples was measured utilizing the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard for calibration. In the chromatographic fractions, the protein content was screened by absorbance at 280 nm.

The hydrolysis time was 60 min for Enzyme I and 30 min for Enzyme II. The hydrolysis rate was linear to time and enzyme concentration. The enzyme activity is given as nmol β -naphthylamine liberated/min/mg protein or ml enzyme solution. The means and the standard deviations were calculated for each group and Student's *t* test was utilized in the statistical correlation.

Experimental design

Dipeptidyl Aminopeptidases I and II were quantified in the testicular tissue during pubertal maturation of the testis. This experiment consisted of seven groups of four animals per group at 10-day intervals between 10 and 70 days of age. The rats in all groups were killed at the same time.

The cryptorchid experiment also involved seven groups. One group of eight animals served as control; the other six groups of four animals per group were killed 2, 5, 8, 10 and 20 days after the induction of cryptorchidism.

In the third experiment, cadmium chloride (0.5 mg/100 g body weight; E. Merck AG, Darmstadt, Germany) was injected subcutaneously into the back. The seven experimental groups with four animals in each were killed 1, 2, 4, 6, 10, 15 and 20 days after treatment and the enzyme activities were compared with those of a control group of eight animals.

RESULTS

Subcellular fractionation

The initial subcellular fractionation indicated that both dipeptidyl Aminopeptidases I and II were localized in the mitochondrial-lysosomal fraction. Much lower activities were encountered in other fractions (Table 1). The sucrose density gradient centrifugation showed both enzyme activities superimposed in fractions which contained the fluoride-sensitive acid phosphatase activity. This confirmed the earlier finding that both these enzymes are lysosomal in origin.

Chromatography

Fractionation of the total testicular tissue homogenate by DEAE-cellulose chromatography resulted in one activity peak for the hydrolysis of Ser-Tyr- β -NA at pH 4.0 with maximum in Fraction 54 (Text-fig. 1). The hydrolysis of Lys-Ala- β -NA showed its maximum in Fraction 65. There was some overlapping between these activity peaks.

Homogenate from isolated seminiferous tubules showed much lower activities, when the same amount of wet tissue was fractionated (Text-fig. 1). The activity peaks were found in coincident fractions with those after total homogenate

Table 1. Relative specific activities of dipeptidyl Aminopeptidase I and dipeptidyl Aminopeptidase II in various subcellular fractions of total and tubular testicular tissue in the rat

Fraction	Aminopeptidase I (Ser-Tyr- β -NA)		Aminopeptidase II (Lys-Ala- β -NA)	
	Total	Tubules	Total	Tubules
Nuclear	0.46	0.64	0.58	0.68
Mitochondrial	2.38	2.16	2.24	2.08
Microsomal	0.62	0.58	0.39	0.55
Soluble	0.57	0.62	0.81	0.70

Specific enzyme activities expressed as a percentage of the total enzyme activity present/the percentage of total protein in the various subcellular fractions.

TEXT-FIG. 1. Elution pattern of dipeptidyl aminopeptidases from the DEAE-cellulose chromatography of homogenates of total testis and seminiferous tubules. The column was eluted with a linear NaCl-gradient (0 to 0.35 M). The protein contents of the 5-ml fractions were scanned by the absorbance at 280 nm (---). The hydrolysis rates of the substrates are given in nmol/ml/min as follows: ▲, Ser-Tyr- β -NA; ■, Lys-Ala- β -NA.

fractionation. It appeared that the hydrolysis of Ser-Tyr- β -NA was relatively much lower than that of Lys-Ala- β -NA.

Optimal pH

The hydrolysis of Ser-Tyr- β -NA by pooled Fractions 48 to 56 showed the maximum hydrolysis at pH 4.5, whereas the hydrolysis of Lys-Ala- β -NA was maximal at pH 5.5 by pooled Fractions 60 to 68 (Text-fig. 2).

Enzyme characteristics

Both enzyme activities showed a linear hydrolysis rate indicating the cleavage of the dipeptide residue at one time and not a sequential splitting from the NH_2 -terminal end. This would result in a curvilinear reaction rate. The same

TEXT-FIG. 2. Effect of pH on the hydrolytic activity of the testicular dipeptidyl aminopeptidases separated by DEAE-cellulose chromatography. Tracings for the enzymes are: $\blacktriangle$, Enzyme I with Ser-Tyr- β -NA as substrate; $\blacksquare$, Enzyme II with Lys-Ala- β -NA as substrate.

fractions also contained the activity of Aminopeptidase B (Vanha-Perttula, 1973a), which, however, is unable to hydrolyse sequentially either Ser-Tyr- β -NA or Lys-Ala- β -NA to result in free β -naphthylamine.

The hydrolysis of Ser-Tyr- β -NA was activated by cysteine, 2-mercaptoethanol and chloride ions, which did not affect the hydrolysis of Lys-Ala- β -NA.

Quantification of enzyme activities under varying experimental conditions

During pubertal maturation of the testis, the hydrolysis of Ser-Tyr- β -NA showed a gradual increase after 20 days of age. During the observation time, no plateau was reached (Text-fig. 3).

The activity of dipeptidyl Aminopeptidase II with Lys-Ala- β -NA as substrate showed a gradual decline in activity throughout pubertal maturation of the

testis (Text-fig. 4). This decrease was not, however, very marked from 20 days of age onwards.

After the induction of cryptorchidism, the seminiferous tubules almost totally lost their spermatogenic elements within 15 days (Vanha-Perttula & Nikkanen, 1972). Enzyme I activity showed an initial decrease in activity, which was significantly different ($P < 0.001$) from the control level at 4 days. Subsequently, a rapid increase ensued which reached a plateau at 15 days (Text-fig. 3).

TEXT-FIG. 3. Hydrolysis of Ser-Tyr- β -NA at pH 4.0 by the testicular homogenate under varying experimental conditions. ○, Enzyme activity during puberty; the age in days is given in the upper abscissa line. ●, Enzyme activity after cryptorchidism; the time after operation is given in the lower abscissa line. △, Enzyme activity after cadmium chloride treatment; the time after treatment is given in the lower abscissa line.

By contrast, Enzyme II activity showed an initial significant rise with peak values at 5 to 8 days ($P < 0.001$) after cryptorchidism. Subsequently, the activity decreased below the control level at 15 to 20 days (Text-fig. 4).

After cadmium chloride treatment, Enzyme I activity showed an initial rise reaching a peak at 5 days ($P < 0.01$), followed by a rapid decline which reached a plateau after 10 days (Text-fig. 3). Enzyme II activity showed an immediate decline following treatment with cadmium chloride (Text-fig. 4). Differences

in the response to the different experimental conditions indicate that these two lysosomal enzymes have differing tissue distributions in the testis and may have varied physiological functions.

TEXT-FIG. 4. Hydrolysis of Lys-Ala- β -NA at pH 5.5 under varying experimental conditions. ○, Enzyme activity during puberty. ●, Enzyme activity after cryptorchidism. Δ, Enzyme activity after cadmium chloride treatment.

DISCUSSION

The present study showed that rat testicular tissue contains two lysosomal dipeptidyl aminopeptidases, which appeared to be identical with those previously characterized from the bovine anterior pituitary (McDonald *et al.*, 1966; McDonald, Leibach, Grindeland & Ellis, 1968; McDonald, Reilly, Zeitman & Ellis, 1968; McDonald *et al.*, 1969). Enzyme I was a halide- and sulphhydryl-dependent acidic dipeptidyl aminopeptidase which has been identified as cathepsin C (McDonald *et al.*, 1969). It has been shown to hydrolyse a variety of dipeptide residues from the NH_2 -terminus of proteins and may also function as a transpeptidase. The Gly-Phe, Ala-Ala and Ser-Tyr residues are readily cleaved from a variety of peptides and proteins (McDonald *et al.*, 1971).

Enzyme II is an acidic dipeptidyl aminopeptidase which is able to split Lys-Ala, Arg-Ala, Leu-Ala, Ala-Ala and Gly-Pro residues from tripeptides (acidic carboxypeptidase) or NH_2 -termini of peptides and proteins (McDonald, Leibach, Grindeland & Ellis, 1968; McDonald, Reilly, Zeitman & Ellis, 1968).

It does not require any dissociable co-factors, but is inhibited by high cation concentrations.

Although lysosomes have been found both in the interstitial (Frank & Christensen, 1968) and seminiferous tubule cells (Tice & Barnett, 1963), the present study indicated that the lysosomal enzyme composition is not necessarily equal in a heterocellular organ like the testis. In addition to the conventional lysosomes, there is another subcellular structure, the acrosomal body, which contains a variety of acid hydrolases closely similar to the lysosomal ones (Allison & Hartree, 1970). There is ample evidence, however, that the acrosomes also contain neutral and basic enzymes (Stambaugh & Buckley, 1969; Zaneveld & Williams, 1970; Ho & Meizel, 1970; Multamäki & Niemi, 1972) and thus cannot be identified as lysosomes. The composition of the lysosomal enzymes in acrosomes has not been totally evaluated and therefore differences can also be anticipated in their relative content of dipeptidyl Aminopeptidases I and II.

Since the lysosomes in the interstitial and spermatogenic cells appear to have a variable composition of hydrolases, their function may also be specialized. A good example is provided by the acrosome which is specialized for the process of fertilization. In this process, one of the dipeptidyl aminopeptidases may be required.

Spermatogenic maturation may offer other specialized functions for acid hydrolases. The Sertoli cells have been implicated as nurturing elements for maturing gametes and as a phagocytic system for the destruction of the residual bodies released from mature spermatozoa. In these functions, lysosomal heterophagocytosis may participate and dipeptidyl aminopeptidases may be required.

In the present study, dipeptidyl Aminopeptidase I appeared to be more concentrated in the interstitial tissues. The marked increase in Enzyme I activity which occurred following the induction of cryptorchidism is consistent with this view, although it does not exclude the Sertoli cells as a possible cellular site. The marked and continuous increase in this activity during puberty indicates a correlation between enzyme activity and the activation of the Leydig cells in steroid production and/or the nurturing activity of the Sertoli cells. The present study did not indicate which cell type prevails in activity. The changes observed in Enzyme I activity closely follow those obtained with the fluoride sensitive acid phosphatase (Vanha-Perttula & Nikkanen, 1972) as well as with Aminopeptidases I and II in similar experimental conditions (Vanha-Perttula, 1973b).

After cadmium chloride treatment, the initial rise of dipeptidyl Aminopeptidase I appears to indicate its activation. A more probable cause for this increase appears, however, to be the relative resistance of the interstitial cells, which contain this enzyme, to the toxic effect of cadmium chloride. After 6 days, when the interstitial cells also show marked degenerative changes, dipeptidyl Aminopeptidase I activity rapidly declines.

The opposite changes of dipeptidyl Aminopeptidases I and II during the initial stages following cryptorchidism, cadmium chloride treatment and during the testicular maturation of puberty substantiate their differential tissue

distribution as well as their possibly varied function. Further studies with isolated testicular cell types are necessary to analyse the cellular variation of enzyme composition and their specialized functions in testicular physiology.

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