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# SPECIFICITY AND DISTRIBUTION OF MAMMALIAN CARNOSINASE

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## Summary

Hog kidney carnosinase (EC 3.4.13.3) was found to have a narrow specificity; it hydrolyzed carnosine, anserine and glycyl-L-histidine, but did not split L-alanyl-L-histidine or homocarnosine. The isoelectric point of this enzyme was 5.8 and its molecular weight was about 84 000. Carnosinase was found to be widely distributed in various tissues of the rat. Uterus, kidney, liver and lung contained high levels of carnosinase, whereas moderate concentrations were found in spleen, heart and brain, with low levels in small intestine, skeletal muscle and stomach, and none in blood.

## Introduction

Mammalian carnosinase (EC 3.4.13.3) was initially characterized in 1949 by Hanson and Smith [1] who found the enzyme to be stabilized and activated by $Mn^{2+}$. Their crude hog kidney carnosinase preparation hydrolyzed carnosine, glycyl-L-histidine, L-alanyl-L-histidine, D-alanyl-L-histidine and glycyl-L-histidinamide. Carnosinase was prepared in highly purified form by Rosenberg [2], who made a careful study of the metal ion interactions of this enzyme [3—5] but did not examine its specificity. Wood [6] made a semiquantitative study of the distribution of carnosine and carnosinase in rat tissues. Perry et al. [7] discovered the metabolic disorder, carnosinemia, and showed that two children with this disease were deficient in serum carnosinase [8]. Recently Murphey et al [9] reported that two electrophoretic forms of carnosinase were present in human kidney, liver, spleen and serum. In the present communication, purified hog kidney carnosinase was found to have an unexpectedly narrow specificity. A quantitative study of the distribution of carnosinase in rat tissues is also presented.

## Materials and Methods

*Carnosinase assays.* For routine assays of hog kidney carnosinase, 50 μl of enzyme solution was added to 100 μl of substrate solution containing 3 mM carnosine (Sigma) dissolved in 25 mM Tris · HCl buffer, pH 7.5. For zero time values, 50 μl of the reaction mixture was added immediately to 0.50 ml water and 1.0 ml of the manual ninhydrin reagent of Moore [10]. After incubation of the reaction mixture for 30 min at 30°C, a second 50-μl aliquot was removed and added to 0.50 ml water and 1.0 ml of the ninhydrin reagent. Colors were developed by the method of Moore [10] and the quantity of substrate hydrolyzed was calculated by reference to a ninhydrin color calibration curve. This curve was prepared from appropriate mixtures of carnosine, β-alanine and histidine representing varying degrees of carnosine hydrolysis. When examining the specificity of carnosinase, the hydrolysis of other substrates (Sigma or Cyclo) was measured by the same procedures. The deacetylation of *N*-acetyl-L-methionine by aminoacylase was also measured by this ninhydrin procedure. Paper chromatography using a ninhydrin spray reagent with previously described techniques [11] confirmed that carnosinase had a dipeptidase action on its substrates, liberating the constituent amino acids.

In studying the distribution of carnosinase, enzyme activity was measured by the fluorimetric procedure of Murphey et al. [9] using 5 mM $MnCl_2$ instead of 0.2 mM $MnCl_2$. Tissues were ground in a mortar with an equal weight of alumina in a 0.3 M Tris · HCl buffer, pH 8.0. After centrifugation, the supernatant liquid was incubated with the carnosine substrate for 60 min at 37°C. Carnosinase did not adsorb on the alumina.

*Carnosinase purification.* DEAE-cellulose and DEAE-Sephadex were used according to the methods of Peterson and Sober [12]. Electrofocusing was carried out in a 110 ml LKB 8100-1 Ampholine column by the procedure of Vesterberg and Svensson [13]. Protein was measured by the method of Lowry et al. [14] employing bovine serum albumin as a standard. The protein of step 1 was precipitated with 10% trichloroacetic acid before assay.

Sephadex G-100 was used for molecular weight determination according to Whitaker [15]; column dimensions were 115 × 1.5 cm and the eluting buffer was 0.05 M acetate, pH 5.8, containing 0.1% Brij 35SP, 0.02% $NaN_3$ and 0.1 mM $MnCl_2$. The column was calibrated using bovine serum albumin (monomer and dimer), ovalbumin, and ribonuclease as marker proteins.

## Results

*Purification of hog kidney carnosinase*

The purification procedure is summarized in Table I. Step 1: 120 g of frozen kidney was diced and then homogenized for 2 min in a blendor with 130 ml of cold distilled water. After standing for 20 min at 4°C, the homogenate was centrifuged for 20 min at 12 000 × *g*. The supernatant was heated for 20 min at 50°C in the presence of 0.01 M $MnCl_2$ [1] and was cooled and recentrifuged at 31 000 × *g* for 20 min at 4°C. Step 2: This crude extract (100 ml) was passed through an 8 × 75 cm Sephadex G-100 column at 23°C. The column was eluted with a 0.02 M Tris · HCl buffer, pH 7.5, containing 0.05 M NaCl.

TABLE I

PURIFICATION OF HOG KIDNEY CARNOSINASE

| Step | Fraction | Volume (ml) | Protein (mg/ml) | Specific activity (μmol/mg per h) | Yield (%) | Purification |
|---|---|---|---|---|---|---|
| 1 | Crude extract | 100 | 18.3 | 3.4 | 100 | 1 |
| 2 | Sephadex G-100 | 460 | 2.6 | 5.0 | 97 | 1.5 |
| 3 | DEAE-cellulose | 122 | 2.33 | 17.0 | 78 | 5 |
| 4 | DEAE-Sephadex | 52 | 0.75 | 81.6 | 51 | 24 |
| 5 | Electrofocusing | 60 | 0.20 | 180 | 36 | 53 |

Step 3: All of the fractions containing carnosinase activity (460 ml) were placed on a 2 × 35 cm DEAE-cellulose column which was equilibrated with 0.01 M Tris buffer, pH 7.5, containing 0.05 M NaCl. The column was washed with this buffer and then a linear gradient of NaCl (0.05—0.6 M in 440 ml of 0.01 M Tris, pH 7.5) was used to elute the carnosinase at 23°C. Step 4: The most active fractions (122 ml) were diluted with 2 volumes of water and placed on a 2 × 13 cm DEAE-Sephadex column which was then washed and eluted as in step 3. Step 5: The most active fractions (52 ml) were concentrated to 10 ml on a Diaflo ultrafiltration membrane (UM20E) and desalted on a 1.5 × 32 cm Sephadex G-25 column. This preparation was subjected to isoelectric focusing at 4°C in a sucrose gradient (pH gradient 4.6—7.0) for 25 h at 550 V and 0.5 mA. The 4.5-ml fractions were dialyzed individually against a 0.02 M Tris buffer, pH 7.5, containing 0.5 mM $MnCl_2$ and were analyzed for carnosinase activity.

Although Murphey et al. [9] found two electrophoretic forms of carnosinase in human tissues, we obtained a single sharp activity peak during isoelectric focusing; the isoelectric point of hog kidney carnosinase was 5.8 ± 0.1. Using the method of Whitaker [15], the apparent molecular weight of this enzyme was found to be about 84 000. The purified enzyme was not affected by 0.5 mM *p*-chloromercuribenzoate or by 0.5 mM dithiothreitol, indicating that it

TABLE II

SPECIFICITY OF HOG KIDNEY CARNOSINASE

| Substrate | Relative rate of hydrolysis (carnosine = 100) | |
|---|---|---|
| | This study | Hanson and Smith [1] |
| Carnosine (β-alanyl-L-histidine) | 100 | 100 |
| Anserine (β-alanyl-1-methylhistidine) | 30 | — |
| L-Alanyl-L-histidine | 0 | 150 |
| Glycyl-L-histidine | 28 | 115 |
| α-Aminobutryl-L-histidine | — | 13 |
| Homocarnosine (γ-aminobutyrylhistidine) | 0 | — |
| L-Histidyl-glycine | 0 | — |
| L-Histidyl-L-histidine | 0 | — |
| *N*-Acetyl-L-histidine | 0 | — |
| *N*-Acetyl-L-methionine | 0 | — |
| *N*-Acetyl-L-leucine | 0 | — |
| *N*-Benzoyl-L-histidine | 0 | — |

TABLE III

DISTRIBUTION OF CARNOSINASE IN TISSUES OF THE RAT

Results are expressed as μmol of carnosine hydrolyzed per g of wet tissue per h and represent the average for eight Wistar rats weighting 275—425 g (four male, four female).

| Tissue | Carnosinase activity | |
|---|---|---|
| | μmol/g per h ± S.D. | Results of Wood [6] |
| Uterus (four rats) | 21.0 | + |
| Kidney | 17.6 ± 6.3 | ++ |
| Liver | 13.8 ± 4.3 | ++ |
| Lung | 11.8 ± 3.0 | ++ |
| Spleen | 7.2 ± 0.6 | + |
| Heart | 6.6 ± 1.6 | ++ |
| Brain | 6.0 ± 1.8 | ++ |
| Skeletal muscle | 3.0 ± 0.6 | trace |
| Small intestine | 3.0 ± 1.2 | ++ |
| Diaphragm | 2.2 ± 0.6 | + |
| Stomach | 1.9 ± 0.2 | + |
| Blood serum | 0 | 0 |
| Blood cells | 0 | 0 |

does not contain sulfhydryl groups or disulfide bonds essential to its activity. Amino acylase (EC 3.5.1.14) accompanied the carnosinase during the first three purification steps, but was separated from it during gradient elution from DEAE-Sephadex. In step 1, heating in the presence of $MnCl_2$ activated carnosinase and precipitated some of the protein in the initial extract, producing a large increase in specific activity.

*Specificity of hog kidney carnosinase*

The purified carnosinase preparation (step 5) was incubated with 11 different dipeptides or acetylated amino acids. In Table II the rates of hydrolysis of these compounds are compared with some of the rate data obtained by Hanson and Smith [1]. It is evident that the crude preparation employed by these authors contained contaminating dipeptidase activity responsible for some of the hydrolysis of glycyl-L-histidine and all of the hydrolysis of L-alanyl-L-histidine.

*Distribution of carnosinase in rat tissues*

The carnosinase contents of 12 tissues of the rat were measured. The results of this study are listed in Table III, where they are compared with the data obtained by Wood [6]. Our data agree roughly with those of Wood [6] except for three tissues: uterus, small intestine and skeletal muscle. Any tissue which contained histidase (EC 4.3.1.3) would give a low apparent carnosinase content, because histidine produced by carnosinase would be deaminated to urocanic acid, which is not measured by the fluorimetric assay. Therefore the histidase activities of these 12 tissues were measured by incubating the extracts with histidine and assaying residual histidine by the fluorimetric technique used in the carnosinase assay; only liver contained appreciable histidase. It was found that 0.2 mM $NH_2OH$ inhibited histidase but not carnosinase. Therefore, liver extracts were analyzed in the presence of $NH_2OH$, which increased their

apparent carnosinase activity by about 25%. None of the tissues displayed histidine decarboxylase activity under the conditions of the carnosinase assay.

## Discussion

Carnosine and anserine are present in high concentrations in the skeletal muscles of vertebrates and homocarnosine is present in brain, but the function of these dipeptides is unknown.

Our knowledge of the specificity of carnosinase rests largely on the work of Hanson and Smith [1]. These authors employed a crude hog kidney enzyme preparation which apparently contained interfering dipeptidases. Davis [16], using a similar preparation, reported that carnosinase hydrolyzes anserine. Rosenberg [3] prepared highly purified hog kidney carnosinase and found that it split glycyl-L-histidine, the only substrate tested other than carnosine. Murphey et al. [9] studied the specificity of human kidney carnosinase. Their crude electrophoresis fractions did not hydrolyze homocarnosine, but split other substrates such as L-alanyl-L-histidine and L-histidyl-glycine with relative activities very similar to those reported by Hanson and Smith [1]. In contrast, the data in Table II indicate that carnosinase has a narrow specificity.

It is usually assumed that carnosinase hydrolyzes homocarnosine (e.g. refs. 17 and 18). In fact, crude hog kidney carnosinase preparations have been employed for the hydrolysis of homocarnosine [19,20]. However, we found that a distinct enzyme hydrolyzes homocarnosine; studies on the purification and characterization of this new dipeptidase will be reported separately.

Hanson and Smith [1] reported that rat liver, kidney and spleen were rich in carnosinase, while the enzyme was not detected in skeletal muscle, heart, hog intestinal mucosa or human uterus. However, we have confirmed the data of Wood [6] who showed that rat heart, small intestine and uterus contained carnosinase. In addition, our results show the presence of this dipeptidase in skeletal muscle, the primary repository for carnosine. It is interesting to note that rat blood contains no detectable carnosinase, whereas human serum contains moderate levels of this enzyme [9].

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