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PORCINE CARBOXYPEPTIDASE B

I. AFFINITY CHROMATOGRAPHY AND SPECIFICITY

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SUMMARY

Porcine carboxypeptidase B (peptidyl-L-lysine hydrolase, EC 3.4.2.2.) was found to possess in addition to its known specificity toward basic substrates an intrinsic activity with specificity similar to that of carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1). Affinity chromatography using a water insoluble derivative of the inhibitor D-Ala–L-Arg was employed in order to establish that the activities toward non-basic substrates are intrinsic and not due to contamination present in the commercial porcine carboxypeptidaes B.

Porcine pancreatic carboxypeptidase B (peptidyl-L-lysine hydrolase, EC 3.4.2.2) is a zinc containing metalloenzyme with a molecular weight of approximately 34 000 which catalyzes the hydrolysis of peptides and esters at the C-terminal bond if the terminal residue is either arginine, lysine or the corresponding α-hydroxyl acids[1-3].

The work to be described grows out of our observation that commercial preparations of porcine carboxypeptidase B have an additional activity similar to that of carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1). Since it has been suggested[3] that the specificity of the two carboxypeptidases is mutually exclusive and that carboxypeptidase B hydrolyses only basic substrates, the possibility that the commercial preparation is contaminated with carboxypeptidase A could not be disregarded. On the other hand, it has been shown[4] that bovine carboxypeptidase B possesses an intrinsic activity with specificity similar to that of carboxypeptidase A. Affinity chromatography seems to be an extremely useful method in order to clarify this point. Therefore, the commercial preparation of carboxypeptidase B was applied to columns of a water insoluble derivative of carboxypeptidase B inhibitors.

Preparation of Sepharose-D–Ala–L-Arg

Activated sepharose[5] (10 ml) was added to a solution of D-Ala–L-Arg (150 μmoles in 10 ml 0.5 M $NaHCO_3$) and the mixture was stirred gently for 20 h at 4°. The resin was then filtered and washed thoroughly with 0.1 M $NaHCO_3$ and water. The amount of peptide coupled to the Sepharose determined by amino acid analysis after

acid hydrolysis was found to be 2.4 μmoles of peptide per 1 ml sepharose. Similarly, other immobilized inhibitors such as Sepharose–D-Arg, Sepharose–ε-aminocaproyl-D-Arg and Sepharose–D-Ala–D-Arg were prepared.

Synthesis of hippuryl-L-argininic acid

Argininic acid (1 mmole) was suspended in anhydrous pyridine (10 ml), and cooled to 4°. Hippuryl chloride (3.5 mmoles) was then added and after 2 h, a dark brown solution was obtained. The mixture was kept at 4° for overnight. The pyridine was removed *in vacuo*, 2–3 lyophilizations were carried out after dissolving the residue in water (each time, 10 ml). The oily residue was then dissolved in water and excess of hippuric acid was removed by extraction with ethylacetate. The solution was finally dried under high vacuum at 50° to yield amorphous solid. High-voltage electrophoresis (3000 V) at pH 1.9 showed the presence of the ester as the major spot and only traces of argininic acid (Sakaguchi). $[\alpha]_D^{20}$ −6.1° (2% in 1 M HCl), lit.[2] $[\alpha]_D^{20}$ −6.2°. The ester was found to be 99% hydrolysed by carboxypeptidase B.

Enzyme activity

Activity toward hippuryl-L-arginine and hippuryl-L-argininic acid was determined spectrophotometrically[3] wherein, the rate of hydrolysis is determined at 254 nm at 25°. Other peptide assays were performed in 0.02 M sodium barbital buffer in 0.1 M NaCl, pH 7.5 and 25°, by the ninhydrin method[4]. The esterase activity was followed[4] by titrating the reaction mixture in solution of 0.04 M NaCl–0.005 M sodium barbital buffer with 0.05–0.01 M NaOH, at pH 7.5 and 25°. The enzyme concentration $1 \cdot 10^{-8}$–$5 \cdot 10^{-8}$ M for esterase activity and $1 \cdot 10^{-7}$–$2 \cdot 10^{-7}$ M for peptidase activity) was chosen to give reliable data for the initial rate of hydrolysis (5–10%). Buffers were extracted with 0.1% dithiozone in carbon tetrachloride to avoid contamination by adventitious metal ions.

When the dipeptide D-Ala–L-Arg was attached to Sepharose 4-B, the resulting material was found to separate commercial carboxypeptidase B (Worthington Biochemical Corp., Freehold, N.J., Code COBDFP) into two fractions. About 40% of the protein was not retained (Fig. 1A) and this fraction was inactive toward hippuryl-arginine, hippurylargininic acid and hippurylphenyl lactate. The second fraction, which was eluted by increasing the salt concentration, had a specific activity 45% higher of the original preparation. From the tested insoluble inhibitors, *i.e.* D-Arg, ε-aminocaproyl-D-Arg, D-Ala–D-Arg and D-Ala–L-Arg, best results were obtained using the latter. As shown in Fig. 1B, β-phenyl propinate, which inhibits competitively the hydrolysis of hippurylarginine, prevents the retention of the active enzyme. Apparently, the soluble inhibitor binds to the carboxypeptidase B, thus, preventing the binding to the insoluble inhibitor. The fact that a small fraction still binds (Fig. 1B) supports this explanation since under these conditions, *i.e.* 0.01 M β-phenyl proprionate, the enzyme is only about 90% inhibited. In addition, that the effect of β-phenyl propionate is not due to the slight increase in ionic strength is illustrated in Fig. 1C, where 0.01 M acetate replaced the β-phenyl propionate. Carboxypeptidase A, slightly succinylated to increase solubility at low salt concentration, was not retained by the D-Ala–L-Arg column. Hence, this column is capable of removing carboxypeptidase A if present in the carboxypeptidase B preparation.

As shown in Table I, the purified carboxypeptidase B eluted from the affinity

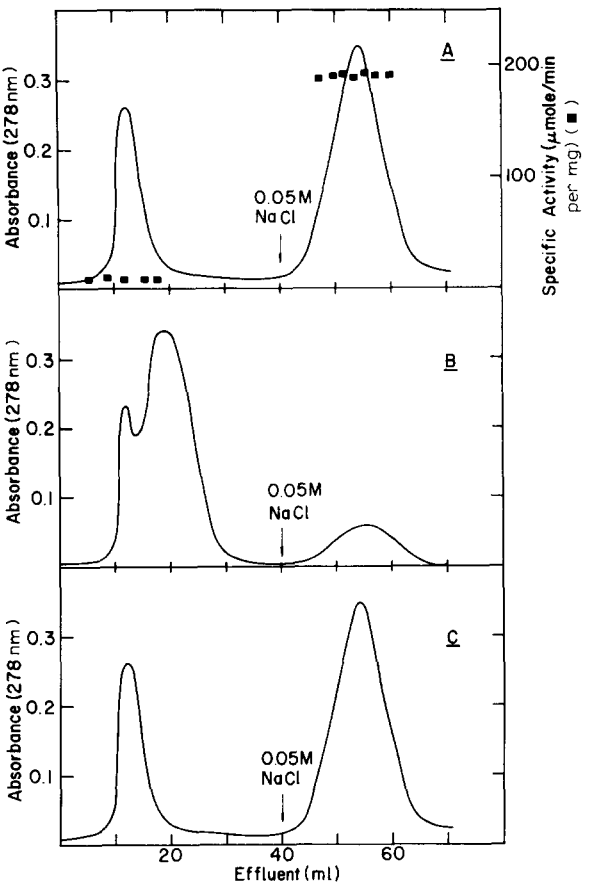

Fig. 1. Affinity chromatography of carboxypeptidase B on a column (1 cm × 15 cm) of Sepharose coupled with D-Ala–L-Arg. 2.5 mg of Worthington carboxypeptidase B (Code COBDFP) were applied to the column. A. The column was pre-equilibrated with 0.025 M Tris–HCl buffer (pH 7.9). The enzyme was eluted after addition of 0.05 M NaCl to the buffer; specific activity in μmoles/min per mg enzyme. B. The column was pre-equilibrated with 0.025 M Tris–HCl, 0.01 M β-phenyl. propionate (pH 7.9). C. The column was pre-equilibrated with 0.025 M Tris–HCl, 0.01 M acetate (pH 7.9).

column was found to hydrolyze in addition to the known basic substrates[3]—hippuryl-arginine and hippurylargininic acid several peptide and esters substrates, *e.g.* Z-$(Ala)_3$, Z-Gly–Phe, Z-$(Gly)_2$–Phe, hippurylphenyl lactate and hippuryl mandelate, known to be specific to carboxypeptidase A action. The hydrolysis of hippurylphenyl lactate by porcine carboxypeptidase B has been also noted in recent reports[11,12].

The kinetic parameters K_m and K_{cat} of carboxypeptidase B action were determined and compared with the values known for carboxypeptidase A (Table I). Similar to carboxypeptidase A, kinetic anomalies, *i.e.* substrates and products causing varying degrees of inhibitions both for the dipeptides and esters, have been observed. Hence, the kinetic parameters were calculated only from the linear phase in the Lineweaver–Burk plots. Nevertheless, the possibility that the extrapolated parameters might be composite terms, should not be discounted. As shown in Table I, the rate of hydro-

TABLE I

KINETIC CONSTANTS FOR PEPTIDES AND ESTERS HYDROLYSIS OF PORCINE CARBOXYPEPTIDASE B AND BOVINE CARBOXYPEPTIDASE A

*Substrate**	*Carboxypeptidase B*		*Carboxypeptidase A*	
	$K_{m(app)} \times 10^4$ *(M)*	K_{cat} *(sec^{-1})*	$K_{m(app)} \times 10^4$ *(M)*	K_{cat} *(sec^{-1})*
Bz-Gly-L-Arg	1.7	96		
	2.1 (ref. 3)	105 (ref. 3)		
Z-Gly-L-Phe	715	14.7	58.3	106 (ref. 6)
Z-$(Gly)_2$-L-Phe	200	39.5	2.5 (ref. 7)	133 (ref. 7)
Z-L-$(Ala)_3$	80	46.5	18 (ref. 8)	70 (ref. 8)
Bz-Gly-L-argininic acid	0.4	220		
	0.4 (ref. 3)	238 (ref. 3)		
Bz-Gly-L-phenyllactic acid	2.8	42	0.88 (ref. 9)	578 (ref. 9)
Bz-Gly-L-mandelic acid	9.1	60	10.9 (ref. 10)	198 (ref. 10)

* Bz, benzoyl; Z, benzyloxycarbonyl.

lysis of the non-basic substrates by carboxypeptidase B is markedly lower than that of carboxypeptidase A which is a result of both an increase in K_m and a decrease in K_{cat}. This is possibly the reason why these activities were not detected previously. The observation that C-terminal residues other than lysine and arginine are hydrolyzed by carboxypeptidase B indicates that caution should be employed when using porcine carboxypeptidase B in sequence studies.

The evidence thus indicates that the specificity of the porcine enzyme is not absolute toward basic substrates and it does not differ in that from the analogous bovine carboxypeptidase B (ref. 4). More detailed examination of the apparent hydrolytic similarities and dissimilarities now in progress might help to decide whether these two specificities of the porcine carboxypeptidase B are mediated by the same active center or whether they are due to different but overlapping centers.

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