Purification and Properties of a New Carboxypeptidase from Citrus Fruit

It has been found that a carboxypeptidase (or a mixture of various carboxypeptidases) occurs in the peel of citrus fruit, for example, of the orange (*Citrus sinensis*), lemon (*Citrus medica*), and grapefruit (*Citrus maxima*). This peptidase differs in specificity and in other properties from the known carboxypeptidases *A* (ref. 1) and *B* (ref. 2) of the pancreas and from the catheptic carboxypeptidase of brain[3]. This citrus peptidase or enzyme mixture, referred to for short as carboxypeptidase *C*, is the first carboxypeptidase to be demonstrated in vegetable tissue. Carboxypeptidase *C* belongs to the catheptic peptidases—a group which, if we extend and re-define the term 'cathepsin', can also be said to include the glycyl–glycine–dipeptidase[4] and leucinaminopeptidase[5] found in leaves.

To obtain a crude carboxypeptidase product, the flavedo (= outermost yellow layer of the peel) was scraped off from 20 kg oranges, homogenized, and extracted with 2·3 per cent sodium chloride solution[11]. Following two fractionations with ammonium sulphate (fractionation I: precipitation at 70 per cent saturation; fractionation II: precipitation at 30 and 70 per cent saturation, active fraction = precipitate at 70 per cent saturation), a crude product (fraction E_1) was obtained after dialysis and lyophilization (fraction E_1 : 2·8 g; C_1 = 0·06 (refs. 6 and 7)). For further purification, the product was submitted to chromatography on '*CM*-Sephadex (*C*–50)' (linear buffer gradient: 0·03–0·3 M sodium acetate buffer or sodium citrate buffer, *p*H 5·3; active fraction E_2 : 90 mg protein[8]; C_1 = 1·3). Repeated fractionation on 'Sephadex *G*–100' yielded a further enriched active fraction (fraction E_3 : 13 mg[8]; C_1 = 4·4).

Investigations carried out so far, chiefly with the crude product fraction E_1, indicate that the enzyme has the following properties and specificity: the *p*H optimum for hydrolysis of all substrates tested is 5·3. Whereas the enzyme is relatively stable (that is, dialysable, lyophilizable) in its impure form (fraction E_1), it is unstable when purified and is rapidly destroyed particularly in the *p*H range 6–10 and at *p*H values of less than 4 (tests with sodium hydroxide, calcium hydroxide, magnesium hydroxide, triethylamine and with hydrochloric acid, acetic acid, respectively). A certain stabilization or activation appears to be achieved by the cations Na^+, K^+, Li^+, and NH_4^+ especially in the range 0·1–1·0 M, whereas the nature of the anion is of no appreciable significance. Phosphate ions display an inhibitory effect[9]. The peptidase is stable at temperatures of up to about 50° C, but rapid inactivation occurs at temperatures above 60° C. The enzymatic activity can be inhibited by Fe^{++} (0·05 M), but not by EDTA (0·05 M, *p*H 5) or *o*-phenanthroline. Diisopropylfluorophosphate (DFP) exhibits an inhibitory effect on fraction E_1 only in high concentration.

The specificity of carboxypeptidase *C* displays the features typical of all carboxypeptidases: hydrolysis of the specific substrate *A*–*B*–*C*–OH between *B* and *C* (*A* = amino-acid residue, peptide residue, acetyl-, carbobenzoxy-, *t*-butyloxy-). The amino-acid in position *C* must have a free carboxyl group; dipeptides (*H*–*B*–*C*–OH–, free α-amino group) are not hydrolysed. D-amino-acids are not split; D-amino-acids in the second position (*B*) from the carboxyl end markedly inhibit the release of the amino-acid at the carboxyl end. Carboxypeptidase *C* splits neutral amino-acids (Leu, Val, and, less rapidly, Gly, Ala), especially aromatic amino-acids (Phe, Tyr), acid amino-acids (Glu, Asp), and basic amino-acids (Lys, Arg, His). A peculiarity of its specificity, however, is its ability to hydrolyse the peptide bond . . . X–Pro —OH in small and large peptides (Table 1).

Table 1. Hydrolysis of Carbobenzoxy Dipeptides with Fraction E_1. (1 h, 30° C, *p*H 5·3; substrate conc. 1 μM/c.c.; enzyme conc. 5 mg fraction E_1/0·5 c.c. [C_1 = 0·06; k_1 = 0·06])

	Percentage hydrolysis*		Percentage hydrolysis
		NH_2	
Z†·Leu–Phe·OH	98	*Z*·Ala–Glu·OH	44
Z ·Pro–Phe·OH	40	*Z*·Gly–Glu·OH	30
Z ·Gly–Leu·OH	35	*Z*·Lys–Asp·OH	37
Z ·Arg–Pro·OH	31	*Z*·Val–Lys·OH	30
Z ·Phe–Pro·OH	20	*Z*·Val–His·OH	26

* Percentage hydrolysis of the amino-acids at the carboxyl end.
† *Z* = Carbobenzoxy-.

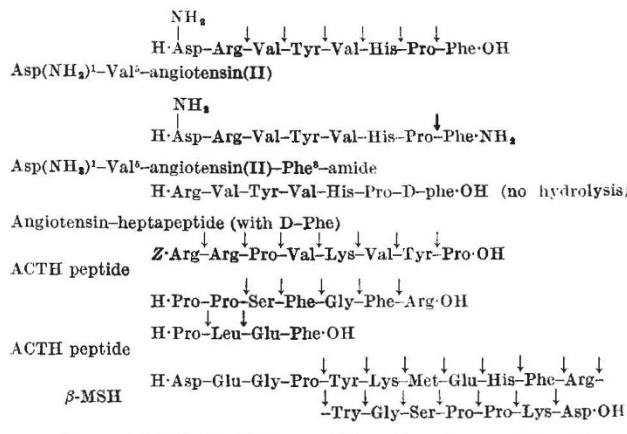

Fig. 1. Hydrolysis of higher peptides with carboxypeptidase *C*

As regards specificity, therefore, carboxypeptidase *C* differs from carboxypeptidase *A* chiefly in that it releases Pro and splits off the acid amino-acids (for example, in β-MSH, Fig. 1) more quickly[10]. Carboxypeptidase *C* possesses esterase activity (fraction E_1 and fraction E_2). as demonstrated in tests on Asp(NH_2)[1]–Val[5]–angiotensin (II)–methyl-ester; the esterase activity in P_1E_2 probably does not come from the acetyl-esterase[11] present in the extracts, since it cannot be inhibited by DFP in the same amount as acetylesterase[11,12]. The crude fraction, E_1, splits off amide groups at the carboxyl end (amidase content), whereas the purified fraction, E_3, does not hydrolyse any amide groups and slowly releases phenylalanine amide in the end position (Fig. 1).

Judging from preliminary experiments, the hydrolysis of *Z*·Leu–Phe·OH is a first-order reaction (fraction E_1 : k_1 = 0·014; C_1 = 0·07 (ref. 7)); for comparison purposes, C_1 values of other substrates were also calculated from the data on the initial rates, although the kinetic characteristics of the hydrolysis of these compounds were not ascertained. These C_1 values were as follows: *Z*·Arg–Pro·OH C_1 (fraction E_1) = 0·0046; C_1 (fraction E_3) = 0·32; *Z*·Phe–Pro·OH C_1 (fraction E_1) = 0·0029; C_1 (fraction E_3) = 0·20; *Z*·Gly–Glu·OH C_1 (fraction E_1) = 0·0045; C_1 (fraction E_3) = 0·31; *Z*·Lys–Asp·OH C_1 (fraction E_1) = 0·0055; C_1 (fraction E_3) = 0·38; *Z*·Val–Lys·OH C_1 (fraction E_1) = 0·0045; C_1 (fraction E_3) = 0·31.

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[6] Measured: hydrolysis of *Z*·Leu-Phe·OH.
[7] C_1 = proteolytic coefficient, k_1 = rate constant (min^{-1}), first-order reaction; substrate conc. 0·001 M, *p*H 5·3, 30° C, enzyme conc. fraction E_1 = 0·2 or 1 mg protein N/c.c.
[8] Protein concentration measured by Folin-Lowry method (*J. Biol. Chem.*, **193**, 265 (1951)).
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[12] There are also other major differences between acetylesterase and carboxypeptidase *C* (*p*H optimum, stability, behaviour during fractionation).