Biochimica et Biophysica Acta, 481 (1977) 594—607

BBA 68094

STUDIES ON THE ENZYMATIC HYDROLYSIS OF POLYGLUTAMYL FOLATES BY CHICKEN LIVER FOLYL POLY-γ-GLUTAMYL CARBOXYPEPTIDASE

I. INTRACELLULAR LOCALIZATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF THE ENZYME

K. NARASIMHA RAO and J.M. NORONHA

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Bombay 400 085 (India)

(Received September 28, 1976)

Summary

Intracellular distribution, purification and properties of a folyl poly-γ-glutamyl carboxypeptidase (peptidyl-L-glutamate hydrolase, EC 3.4.12.10) from chicken liver have been investigated. The post-nuclear particulate and cell supernatant fractions showed activity. The particulate enzyme exhibited characteristics suggestive of its lysosomal origin; on solubilization, however, it cannot be distinguished from the cell cytosol activity. The bulk enzyme was purified about 80-fold to apparent homogeneity by 50—90% ammonium sulfate fractional precipitation, dialysis and chromatography on a 'mixed column' of Sephadex G-100 superimposed on CM-Sephadex C-50. The purified enzyme behaved homogeneously on Sephadex G-100 chromatography, sucrose density gradient centrifugation analyses and polyacrylamide gel electrophoresis. However, polyacrylamide gel electrophoresis in the presence of mercaptoethanol dissociated the native enzyme into two separable isoenzymic components.

The enzyme exhibits two pH optima (4.1 and 5.2) and a temperature optimum of 35—40°C. The reaction is linear for 20 min. The enzyme sequentially cleaves the terminal γ-glutamyl residues of polyglutamylfolates, finally releasing a monoglutamyl end-product. An apparent K_m value of $0.83 \cdot 10^{-6}$ M and a V of 1.50 mmol/min were determined for N^5-methyltetrahydropteroyltetraglutamate with 0.20 mg enzyme protein/ml reaction. The enzyme is substan-

---

Enquiries may be addressed to: Dr. J.M. Noronha, Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Bombay 400 085, India.

This work is part of a thesis submitted by K.N. Rao to the Aligarh Muslim University in partial fulfilment of the requirements for the award of the Ph.D. degree.

Abbreviation: DEAE-cellulose, diethylaminoethyl cellulose.

tially stimulated in the presence of mercaptoethanol, Na^+, Mn^{2+} and low concentrations of denaturing agents (urea). Citrate potently inhibits and phosphate inactivates the enzyme.

Introduction

Enzymes which cleave the naturally occurring folyl poly-γ-L-glutamates (loosely termed as 'folyl conjugases') have recently been assigned the name glutamate carboxypeptidases (peptidyl-L-glutamate hydrolase, EC 3.4.12.10). The activity is widely distributed throughout the phylogenetic spectrum [1] and the enzymes studied so far display a wide variety of characterized properties. Some mammalian conjugases, presumably of lysosomal origin, which include enzymes from hog kidney [2,3], leukocytes [4], guinea pig intestine [5], human liver [6] and placenta [7], rat bone marrow [8] intestine [9] and liver [10] have pH optima in the range of 4.0—5.0. However, the chicken intestinal enzyme complex [11], the chicken pancreas enzyme [12] and the enzyme from *Flavobacterium polyglutamicum* [13] are optimally active in the alkaline pH range. Partially purified preparations from chicken pancreas [14], hog kidney [3] and human liver, intestine and brain [15] are characteristically carboxypeptidase which sequentially cleave the terminal γ-glutamyl peptide. However the bovine liver [16] and a chick intestinal enzyme [11] cleave the internal bonds and thus are more appropriately hydrolases. A carboxypeptidase specific for pteroyl γ-diglutamate and a γ-oligoglutamyl aminopeptidase are also associated with the chick intestine conjugase complex [17]. While the human liver enzyme failed to hydrolyze α-bonds [6], the chick pancreas enzyme could hydrolyze them at half the rate at which the γ-glutamyl bonds were cleaved [18]. In addition to the common requirement for the maintenance of reduced sulfhydryl groups, the bovine hepatic enzyme requires Zn^{2+} for stability and is a glycoprotein [16].

Despite the varied characteristics of these enzymes from different sources, their main function seems to be in aiding folate absorption and transport through digestion of the naturally occurring polyglutamyl folates. It is also suggested that the enzyme could serve to release free glutamic acid for the γ-glutamyl cycle-linked transport of amino acids [19] and the synthesis of γ-carboxyglutamic acid-containing proteins regulating the mineralization of calcified tissues [20].

In this paper we report on the nature of a folyl γ-glutamyl carboxypeptidase derived from chicken liver - its intracellular localization, a method convenient for purifying the activity to homogeneity, some of its properties, kinetics and mechanism of action. The paper that follows reports on structural studies with the enzyme [21].

Materials and Methods

Chemicals

All the chemicals used were of reagent or analytical grade. N^5-formyltetrahydrofolic acid was obtained from Lederle Laboratories Division, American

Cyanamide Company, New York. DEAE-cellulose, L-ascorbic acid and *Candida utilis* (Torula yeast) were products of Sigma Chemical Company, St. Louis, Missouri. 'Folic acid casei medium' was purchased from Difco Laboratories, Detroit. CM-Sephadex C-50 and Sephadex G-100 and G-15 were purchased from Pharmacia Fine Chemicals, Uppsala. "Ultra Pure" grade sucrose, urea and guanidine hydrochloride were purchased from Mann Research Laboratories, Orangeburg, New York. Tetramethylethylenediamine, acrylamide and methylene bisacrylamide were obtained from Eastern Organic Chemicals, Rochester.

Preparation of subcellular fractions

Male white leghorn chickens (about 2—3 months old) were fasted overnight, stunned and decapitated. The liver was excised, washed with ice-cold isotonic 0.25 M sucrose and minced. A 10% (w/v) homogenate was prepared at 0—4°C in 0.25 M sucrose using a Potter-Elvehjem-type Teflon pestle homogenizer run at a speed of 1000—1200 rev./min. The isolation of subcellular fractions (heavy and light mitochondria, microsomes and the cytoplasmic supernatant) was carried out by differential centrifugation at 0—4°C essentially as described by Werkheiser and Bartley [22]. All the particulate fractions were washed once in 0.25 M sucrose and resedimented at the specified speeds. Finally each fraction was suspended in 20 mM potassium phosphate buffer (pH 7.0) containing 14.3 mM β-mercaptoethanol.

Marker enzyme assays of subcellular fractions

Succinoxidase activity characteristic of the mitochondrial fraction was assayed according to Potter [23]. Microsomal glucose-6′-phosphatase activity was determined by the method of Swanson [24]. Cathepsin and the acid phosphatase activities, estimated according to the methods described by Gianetto and de Duve [25] represented typical lysosomal enzymes.

Purification of the bulk enzyme

A 10% (w/v) homogenate prepared at 0—4°C in 20 mM potassium phosphate buffer (pH 6.0) was centrifuged at 8500 × *g* for 15 min and the total supernatant (containing the particulate and the cytosol enzyme) was fractionated with the ammonium sulfate at 0—2°C. The active 50—90% ammonium sulfate precipitate was collected by centrifugation at 10 000 rev./min for 10 min and dissolved in the 20 mM phosphate buffer (about 1.0 ml per g of the starting material). At this stage the lyophilized powder could be stored at —20°C without loss of activity for at least 10 days. After reconstitution it was dialyzed against chilled (4°C) distilled water for 18 h.

CM-Sephadex C-50-Sephadex G-100 'mixed column' chromatography

A slurry of CM-Sephadex C-50, equilibrated in 20 mM phosphate buffer (pH 6.0) was allowed to settle as a 15 cm bed in 2.1 × 60-cm Pyrex glass columns. Sephadex G-100 (equilibrated in the same buffer) was then overlayered as a thin slurry till the combined bed height of the 'mixed column' measured 50 cm. The dialyzed enzyme preparation was fractionated by elution with the equilibrating buffer. Higher phosphate concentrations caused degradation of the enzyme into several less active fractions.

Protein determination

Protein content of the subcellular and the purified enzyme fractions was measured by the method of Lowry et al. [26].

Assay of folyl γ-glutamyl carboxypeptidase activity

Mixed folylpolyglutamates extracted from Torula yeast, *C. utilis* served as the substrate in most of the experiments. A 10% (w/v) Torula yeast extract was prepared in 1% ascorbate containing 0.1 M acetate buffer (pH 4.1) at 70°C for 30 min [27]. Folate contents of clarified extract before and after conjugase digestion were 0.6 and 6.5 μg/g respectively.

The incubation mixture for the γ-glutamyl carboxypeptidase reaction consisted of 0.1 M acetate buffer containing 1% ascorbate (pH 4.1), 0.1 ml of 10% Torula yeast extract, 0.2 mg protein of the isolated subcellular or the purified enzyme preparations and 14.3 mM β-mercaptoethanol in a total volume of 1 ml. The reactions initiated by the addition of enzyme, were incubated at 37°C for 20 min. Appropriate blanks were run simultaneously. Reactions were terminated by boiling the incubation mixtures for 5 min in a water bath. 1% ascorbate (pH 6.0) was added to make up the final volumes to 5 ml. Reaction mixtures were then assayed microbiologically for the enzymatic release of folate activity after suitable dilutions using *Lactobacillus casei* (ATCC 7469) as the test organism, the Difco basal medium and synthetic N^5-formyltetrahydrofolic acid as a reference standard. Corrections were made for the inactive isomer. The released freely assayable monoglutamylpteroate derivatives were measured as an index of carboxypeptidase activity. Assay conditions which prevented degradative changes due to oxidation, were employed [28].

Tests for homogeneity of the purified enzyme

Chromatography on Sephadex G-100 and 5—15% sucrose density gradient centrifugation analyses were employed as criteria for enzyme homogeneity. Additionally the purity was checked on polyacrylamide gel electrophoresis [29] in the absence and presence of β-mercaptoethanol. The bands were isolated [30] and tested for activity.

End product analyses

(a) Preparation of a defined folylpolyglutamate substrate from Torula yeast. A pteroylpolyglutamate fraction from Torula yeast obtained in Fraction 21 on preparative DEAE-cellulose chromatography as described earlier [31] was lyophilized and partially desalted by repeated precipitations in ethanol/water (3 : 1) and concentration on a Buchler flash evaporator. It was suspended in 5 ml of 1% ascorbate (pH 6.0) and 1 ml of this preparation (about 50 μg of folate activity) was further desalted by rechromatography on Sephadex G-15 (1.3 × 150 cm) [32] collecting 6 ml fractions with distilled water as the eluant. The eluted phosphate-free pteroylpolyglutamate (Fractions 14—17) was lyophilized to dryness and dissolved in 1.0 ml of 0.1 M acetate buffer (pH 4.1) containing 1% ascorbate. This preparation (identified as N^5-methyltetrahydropteroyltetraglutamate, see Results) served as a defined substrate for characterizing the products of the enzymatic reaction.

(b) Characterization of the products of the reaction. Aliquots from a bulk

reaction mixture were withdrawn at various time intervals, the reactions terminated and the products formed were characterized by chromatography on identical analytical DEAE-cellulose columns (1.2 × 20 cm) essentially according to the method of Silverman et al. [33]. The eluted fractions were assayed for folate activity with *L. casei* as the test organism. Phosphate content of the fractions was determined according to the method of Sumner [34].

Results

(A) Subcellular distribution of the enzyme

Results presented in Table I show the distribution profiles of folyl γ-glutamyl carboxypeptidase activity in the isolated subcellular fractions. The purity of the isolated fractions is indicated by the corresponding marker enzyme activity profiles: (a) succinoxidase for the mitochondrial preparation, (b) cathepsin and acid phosphatase representing the lysosomal fraction and (c) glucose-6′-phosphatase, the microsomal component. As is seen in Table I, the marker lysosomal enzymes are found to be distributed in a manner which closely paralleled the γ-glutamyl carboxypeptidase activity profile. They were almost equally distributed in our preparation of the mitochondrial, microsomal and supernatant fractions.

No attempts were made to isolate the lysosomes which sedimented with our mitochondrial and microsomal fractions. Instead, after discarding the nuclei and unbroken cells (650 × g, 10 min) the homogenate in 0.25 M sucrose was spun at 105 000 × g for 1 h and the particulate enzyme, presumably associated with the sedimented lysosomes, was studied in comparison with the soluble enzyme in the post-105 000 × *g* supernatant. There was a stepwise increase in the released particulate γ-glutamyl carboxypeptidase and acid phosphatase activities after freezing and thawing up to 7 times (Fig. 1). In contrast, the cell supernatant enzyme activity did not increase after freezing and thawing. Since

TABLE I

SUBCELLULAR DISTRIBUTION OF ENZYME ACTIVITIES OF CHICKEN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE AND THE OTHER MARKER ENZYMES

Enzyme activities were assayed as described and referred to in the text. The values represent activities per mg protein and are based on averages for three separate reactions. The activities for cathepsin and acid phosphatase were measured for 10-min reactions whereas those for succinoxidase, glucose-6′-phosphatase and folyl γ-glutamyl carboxypeptidase were measured for 20-min reactions.

Enzyme source	Succinoxidase (μl O_2 evolved)	Cathepsin (nmol tyrosine released)	Acid phosphatase (nmol P_i released)	Glucose-6′-phosphatase (nmol P_i released)	Folyl γ-glutamyl carboxypeptidase (ng folate released)
Homogenate	3.24	198	126	563	144
Nuclei	2.93	32	57	38	19
Heavy mitochondria	15.52	342	474	244	326
Light mitochondria	8.47	292	366	487	210
Microsomes	1.70	72	150	1050	116
Supernatant (cell sap)	1.40	36	63	113	173

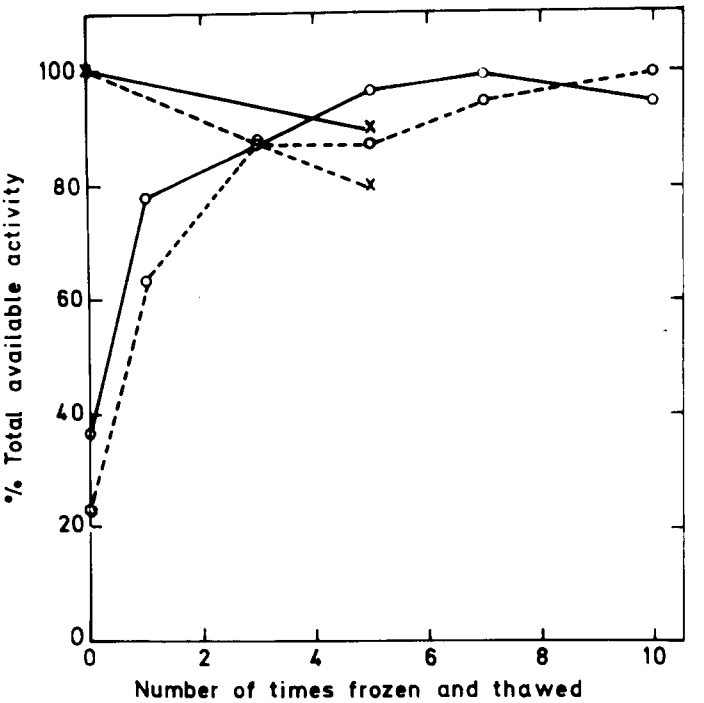

Fig. 1. Release after freezing and thawing of chicken liver γ-glutamyl carboxypeptidase. Maximal activity (after 7 times freezing and thawing) was taken as 100%. γ-Glutamyl carboxypeptidase activity of particulate o———o, acid phosphatase activity of particulate o- - - - -o; γ-glutamyl carboxypeptidase activity of supernatant X———X, acid phosphatase activity of supernatant X- - - - -X. The enzyme activities were assayed as referred to in the text.

the supernatant activity itself could have been released from the lysosomes during fractionation, these activities were studied separately for comparison.

The particulate enzyme was solubilized in 20 mM phosphate (pH 7.0) by rigorous homogenization at 0—4°C. The resultant homogenate was centrifuged at 10 000 rev./min for 10 min and the active supernatant collected. The cell supernatant and the solubilized particulate enzyme were precipitated between the 50—90% ammonium sulfate concentration, had an identical pH optimum of around 4.1 and were similarly stimulated at an optimal concentration of 14.3 mM β-mercaptoethanol (untabulated results). Their separate elution profiles after chromatography on the Sephadex G-100 and the 'mixed' CM-Sephadex C-50-Sephadex G-100 columns were also identical (untabulated results). Since we were unable to distinguish between the particulate and the supernatant activities, the bulk enzyme purification and further studies were carried out with the combined particulate and supernatant fractions.

(B) Purification of the bulk enzyme

The purification procedure and a typical preparation of folyl γ-glutamyl carboxypeptidase from chicken liver are summarized in Table II. The enzymatically active fraction obtained after chromatography on the 'mixed column' of CM-Sephadex C-50-Sephadex G-100 (see Fig. 2) represented a 79-fold purification with a 3% recovery of the initial activity of the crude extract. The 'mixed col-

TABLE II

PURIFICATION OF CHICKEN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE

The post nuclear cell supernatant was subjected to ammonium sulfate fractionation, dialysis and finally chromatography on a combined CM-Sephadex C-50-Sephadex G-100 column as detailed in the text. The values presented for the final step purification represent estimations for the symmetrical activity peak as eluted in Fractions 17—19 in Fig. 2. Specific activity refers to freely assayable folate released during the 20-min reaction.

Purification step	Volume (ml)	Protein (mg/ml)	Specific activity (ng folate released/mg protein)	Yield (%)	Purification (-fold)
I Crude homogenate	400	60.0	3.7	100	(1)
II Supernatant (>8500 × g)	230	35.0	8.9	80	2.4
III 50—90% ammonium sulfate fraction	33	16.5	27.0	17	7.3
IV CM-Sephadex C-50-Sephadex G-100 fraction	10.5	0.73	293.0	3	79.0

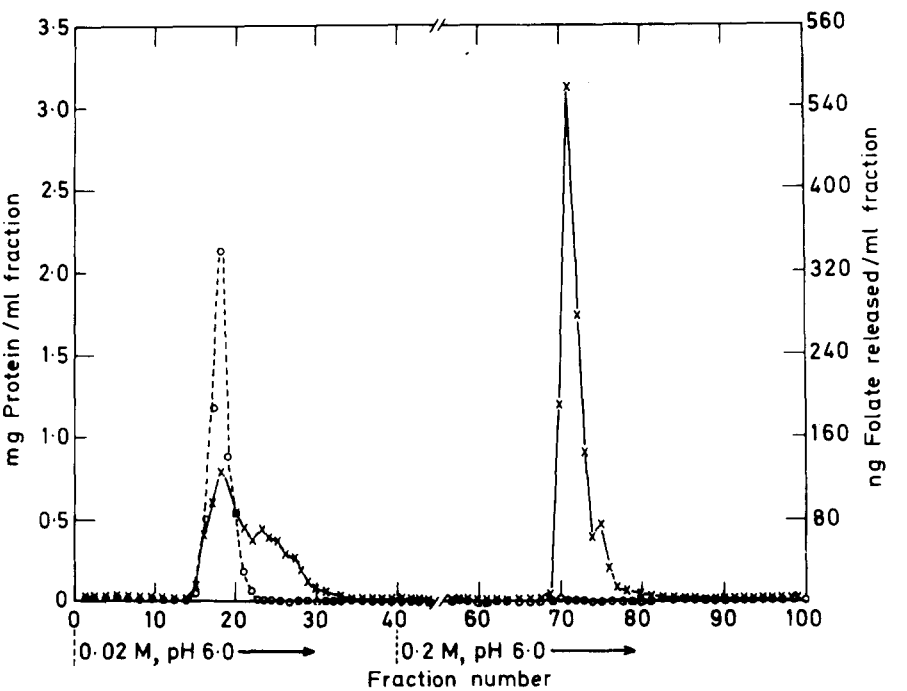

Fig. 2. Chromatography of chicken liver γ-glutamyl carboxypeptidase on a 'mixed column' of CM-Sephadex C-50 and Sephadex G-100. The 'mixed column' was prepared as described and 50—60 mg enzyme (3—4 ml) after dialysis was layered on the column and eluted with 20 mM phosphate buffer (pH 6.0). 40 3.5 ml fractions were collected at 0—4°C. The enzyme eluted out as a homogeneous peak in Fractions 17—19. A further 210 ml of eluate collected with 0.2 M phosphate buffer (pH 6.0) as the eluant served to recover most of the inactive protein (Fractions 69—70). After sampling for protein determinations each fraction was made up to contain 14.3 mM β-mercaptoethanol before enzyme assay. Protein (X———X) and γ-glutamyl carboxypeptidase activity (○- - - - -○) were determined as described.

umn' chromatographic fractionation procedure employed brought about the the combined resolutions effected by molecular sieve and ion exchange chromatography in a single purification step. The enzyme protein eluted out symmetrically between 53 and 77 ml of the effluent with about 16% recovery of activity. The bulk of the protein eluted out broadly in three major peaks with 85% recovery. Testicular hyaluronidase has been prepared earlier by a somewhat similar procedure [35].

In other experiments employing 0.1 M phosphate buffer for the chromatographic elution, it was found that the symmetrical enzyme peak obtained with 20 mM phosphate eluant (Fig. 2) has dissociated into four protein peaks (in Fractions 14, 28, 34 and 51). Further when the buffer concentration was raised to 0.5 M, the enzyme had fragmented into six lower-molecular-weight peaks (Fractions 15, 18, 25, 33, 41 and 47) with proportionate loss of enzyme activity. This observation may explain our failure in earlier attempts to purify the

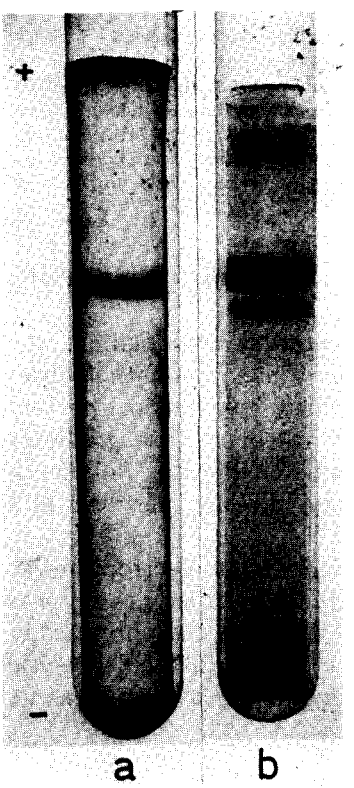

Fig. 3. Polyacrylamide gel electrophoresis of purified chicken liver γ-glutamyl carboxypeptidase. Analytical gels (7.5%) prepared [29] in 20 mM sodium phosphate buffer (pH 7.0) were layered over with about 35 μg (30 μl) of the protein. Electrophoresis was carried out in a 'Canalco Model 6' set up using Bromophenol Blue as the tracking dye. The net current applied was 8 mA/gel at 80 V for about 3 h. The gels were run in cold at 4°C. The direction of the migration of the ions was from positive to negative. In (a) no mercaptoethanol was present while in (b) 14.3 mM β-mercaptoethanol was present in the protein sample, the gel and the electrophoresis buffer. The gels were stained in 1% Coomassie Brilliant Blue and destained in 7% acetic acid.

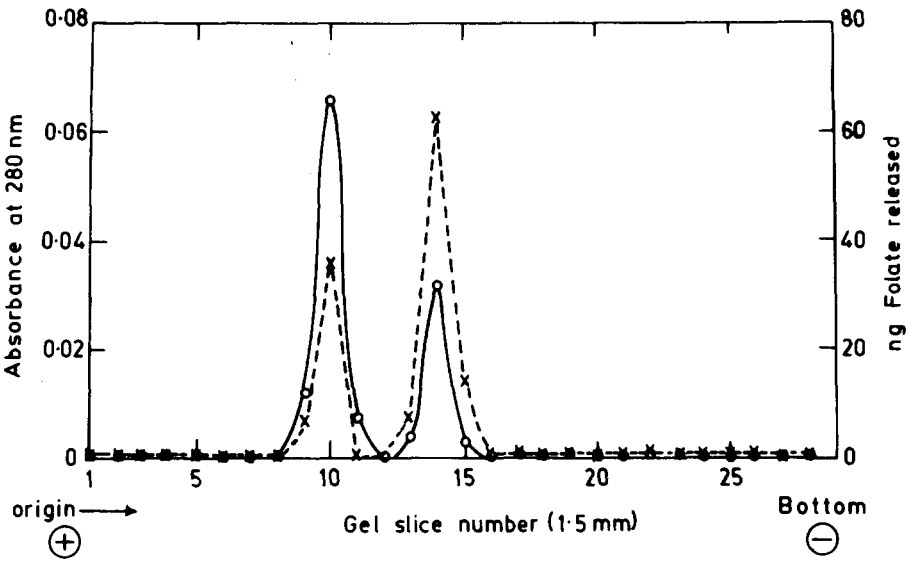

Fig. 4. Enzyme activities of purified chicken liver γ-glutamyl carboxypeptidase bands obtained on disc gel electrophoresis in the presence of β-mercaptoethanol (Fig. 3b). The protein bands were eluted from the gels essentially as described by Smith [30]. The gels were removed from the tubes and frozen at −20°C for an hour. The frozen gels were sliced transversely into 1.5-mm thick segments and suspended in 1.3 ml of 0.1 M ice-cold acetate buffer (pH 4.1) overnight at 0—2°C. The gel slices were dispersed by homogenization and centrifuged. The supernatants were assayed for protein and γ-glutamyl carboxypeptidase activity as described. Values for absorbance at 280 nm (○———○) and γ-glutamyl carboxypeptidase activity (X- - - - -X) are for each slice after elution.

enzyme as well as the reported failures of other investigators to purify the enzyme from other sources [2].

The homogeneity of the purified enzyme preparation obtained on the 'mixed column' was tested by three different criteria. The enzyme when chromatographed on Sephadex G-100, eluted out as a single symmetrical protein peak. Sedimentation analyses of the purified enzyme on sucrose gradients resulted in a single symmetrical protein peak (untabulated results). Polyacrylamide disc gel electrophoresis of the purified enzyme similarly resulted in a single homogeneous protein band (see Fig. 3a).

While the enzyme was homogeneous on polyacrylamide gel electrophoresis, in the added presence of mercaptoethanol (14.3 mM) the purified protein was resolved into two components (Fig. 3b). Both these electrophoretically resolved fractions were enzymatically active (Fig. 4) with different specific activities, the more positively charged fraction being twice as active as its counterpart.

(C) Properties of the enzyme

pH optima and the kinetic properties. The native enzyme has a sharp major pH optimum at pH 4.1 and a broader second pH optimum around pH 5.2. About 54% of the activity at pH 4.1 was observed around pH 5.2. Activity is linear up to 20—30 min of reaction with a broad temperature optimum between 35 and 40°C. Thermal inactivation experiments showed that only 50% of the original activity was lost after 110 s of exposure to a temperature of

55°C. The optimal concentration of the purified enzyme ranges between 0.15 and 0.20 mg protein per ml of the reaction system. Extrapolation of the double reciprocal plot of substrate concentrations between the range 0.14 and 1.42 μM and the corresponding initial velocities showed typical Michaelis-Menten kinetics with 0.20 mg enzyme protein per ml reaction and gave a K_m value of $0.83 \cdot 10^{-6}$ M and a V of 1.50 mmol per min for N^5-methyltetrahydropteroyltetraglutamate.

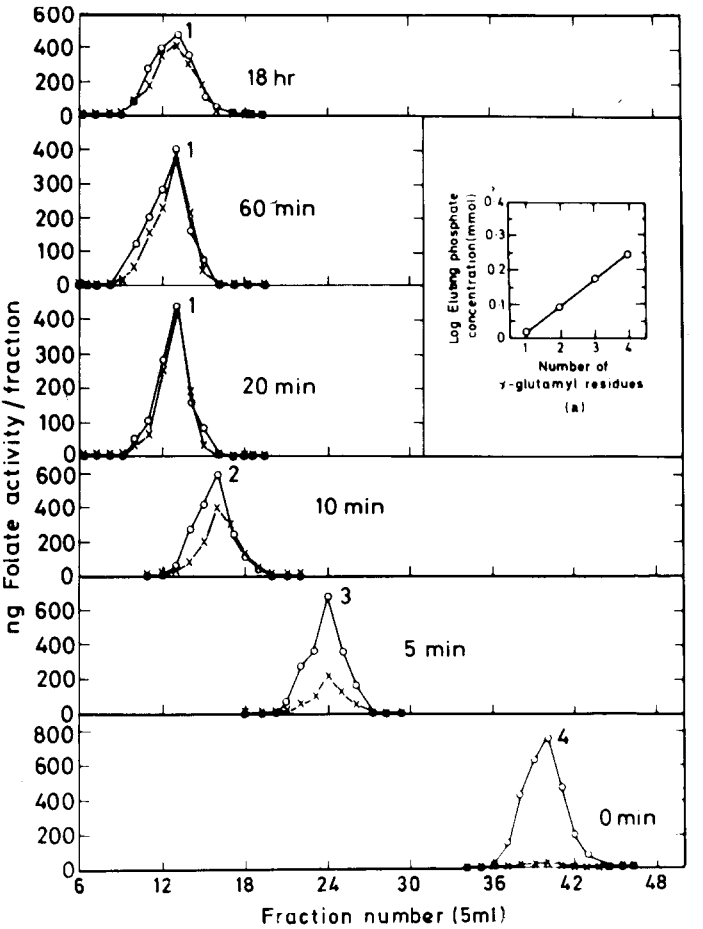

Fig. 5. Analytical DEAE-cellulose chromatograms of individual reaction mixtures stopped at various time intervals for analysis of the end-product of the γ-glutamyl carboxypeptidase reaction. Bulk reaction consisting of 8.0 ml 0.1 M acetate buffer containing 1% ascorbate (pH 4.1), 1.0 ml of the purified yeast pteroylpolyglutamate preparation (0.85 μM) and 1.0 ml of the purified chicken liver enzyme (1.82 mg) containing 14.3 mM β-mercaptoethanol in a final volume of 10.0 ml was initiated by the addition of enzyme and incubated at 37°C. Aliquots of 1.5 ml each were removed at 0, 5, 10, 20, 60 min and 18 h time intervals and the reaction terminated by boiling the tubes at 100°C for 5 min. To each reaction mixture 0.15 ml of 10% ascorbate (pH 6.0) was added and the reaction mixtures individually analyzed on analytical DEAE-cellulose columns for the products formed as described earlier [32]. *L. casei* folate response of the eluted fractions of the different reactions prior to (X———X) and after γ-glutamyl carboxypeptidase digestion [32] (○———○) are plotted for each fraction. In Fig. 5(a) the logarithm of the phosphate concentration in the eluant is plotted against the number of glutamate residues in the corresponding folate derivative eluting out.

End-product analyses of the enzymatic reaction. The folate profiles of aliquots of incubating reaction mixture from the bulk mixtures using the purified N^5-methyltetrahydropteroyltetraglutamate as a defined substrate were analyzed on a analytical DEAE-cellulose columns [33] and are presented in Fig. 5. The 0 min reaction mixture displayed a single folate peak in Fraction 40 representing the unaltered substrate. The location of this peak on the analytical chromatogram, its exclusive activity for *L. casei*, the phosphate concentration at which it is eluted (Fig. 5a) and the 25-fold increase in the *L. casei* response after carboxypeptidase digestion are consistent with its identification as N^5-methyltetrahydropteroyltetraglutamate.

The folate response of the 5- and 10-min reactions appears in Fractions 24 and 16, suggesting that the initial pteroyltetraglutamate was being progressively cleaved to pteroate derivatives previously identified as the tri- and diglutamates respectively [31]. The phosphate concentration at which these compounds elute out as well as the increasing microbiological growth response prior to carboxypeptidase digestion confirm the identity of these compounds. It is next seen that after 20 min of the enzymatic reaction the folate activity peak appears in Fraction 13, where authentic N^5-methyltetrahydrofolate elutes [31], thus suggesting complete hydrolysis to the freely assayable pteroylmonoglutamyl derivative. No further change in folate activity elution profile was observed for reaction beyond 20 min (60 min and 18 h) at which time interval the reaction was essentially complete. Thus the results conclusively show that purified chicken liver γ-glutamyl carboxypeptidase is capable of cleaving stepwise the terminal γ-glutamyl peptide bond of the methyltetrahydropteroyltetraglutamate to finally release the corresponding monoglutamate without alteration of the attached 1-C residue.

A relationship between the number of glutamate residues and the log of the phosphate concentration of the eluting buffer has been observed earlier [36]. Chan et al. [37] have also quantified the observed partial microbiological growth responses to the complexity of the corresponding polyglutamyl folate derivatives.

Effect of mercaptoethanol, cations, anions and denaturing agents. Greater than a 2-fold stimulation of the enzyme activity was observed at an optimal concentration of 14.3 mM β-mercaptoethanol. This stimulation was lost after dialysis against water at 4°C for 18 h. The decreased activity could be restored on addition of 14.3 mM mercaptoethanol to the dialyzed enzyme.

The order of cations which stimulate enzyme activity is in the sequence: Mn^{2+}, $Na^+ > K^+ > Ca^{2+} > Mg^{2+}$. The ionic concentrations at which γ-glutamyl carboxypeptidase was maximally stimulated differed from ion to ion. Thus a 16-fold higher concentration of Mn^{2+} (1.6 M) as compared to Na^+ (0.1 M) was necessary for an 8-fold stimulation over the initial activity. Li^+, NH_4^+, Cu^{2+}, Zn^{2+}, Fe^{2+} and Ba^{2+} did not affect enzyme activity. CO_3^{2-} and I^- activated γ-glutamyl carboxypeptidase slightly whereas citrate, SO_4^{2-}, $S_2O_3^{2-}$, CO_3^{2-} and I^- inhibited the enzyme in that order. Though citrate was the most potent inhibitor (100% inhibition at 0.4 M concentration), a slight stimulatory effect was observed below 0.1 M citrate. It has already been pointed out that phosphate concentration above 20 mM promoted the autolysis of the enzyme with loss of activity.

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON THE CHICKEN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE ACTIVITY

The specific activity of the enzyme in the presence of β-mercaptoethanol was taken as 100. The effect of the various additions was studied in the presence of β-mercaptoethanol. Cations were present as chlorides, citrate as sodium salt and phosphate as the potassium salt. Enzyme incubations in the presence of various specified compounds were carried out in a final volume of 1.0 ml and the enzyme activity was assayed as described in the text. The concentrations at which the compounds were added did not affect the microbiological assay.

Conditions	Concentration	Relative activity
— β-Mercaptoethanol	—	43
Control (+ mercaptoethanol)	(14.3 mM) *	100
+ Mn^{2+}	1.6 M **	817
+ Na^{+}	100 mM **	717
+ Phosphate	100 mM	83
+ Citrate	60 mM	114
	100 mM	34
+ Urea	0.55 M **	503
	3 M	70
+ Guanidine · HCl	50 mM **	178
	1 M	13
+ Thiourea	50 mM	73

* Refers to the concentration of β-mercaptoethanol which was found to be optimal and which was also present in the rest of the incubations.

** Refers to concentrations at which maximal activity was observed.

Presence of 0.55 M urea in the incubation mixture markedly (about 5-fold) stimulated the chicken liver enzyme. Urea concentrations greater than 2.0 M resulted in a marked inhibition of the enzyme activity. 50 mM guanidine hydrochloride stimulated the enzyme about 2-fold while concentrations greater than 0.1 M resulted in a marked inhibition of the activity. Thiourea however potently inhibited activity over the entire range studied (25 mM to 1.0 M). Effect of various compounds on the γ-glutamyl carboxypeptidase activity is summarized in Table III.

Discussion

The results presented indicate that the chicken liver folyl γ-glutamyl carboxypeptidase activity is distributed mainly in the post nuclear particulate fraction and in the soluble cell sap. The stepwise release of bound activity on freezing and thawing, and acidic pH optima are suggestive of the lysosomal origin of the particulate enzyme. However no carbohydrate covalently linked to the enzyme protein could be detected by the anthrone procedure [38]. The observed identical properties of the supernatant and the solubilized particulate enzyme such as their ammonium sulfate fractional pattern, their similar pH optima and thiol group requirement as well as their identical chromatographic behaviour on the Sephadex G-100 and the CM-Sephadex C-50-Sephadex G-100 mixed column suggested that these two subcellular carboxypeptidase activities may have a common origin.

The 'mixed column' technique is a particularly convenient procedure which served to resolve the bulk protein and provide a 79-fold homogeneous enzyme preparation. On gel electrophoresis in the presence of β-mercaptoethanol, however, the enzyme is resolved into two polypeptide components, both enzymatically active. Structural studies in the following paper [21] indicate that the native enzyme is constituted of two polypeptide chains, linked by a disulfide bridge.

Longer chain poly γ-glutamylfolates are preferably hydrolyzed by the enzyme under study. Time course analyses of the products of the reaction indicate that only the terminal γ-glutamyl bond is cleaved and the exopeptidase activity proceeds to completion before the next lower poly γ-glutamylfolate is attacked. Internal glutamyl bonds are not cleaved. In contrast a multiple enzyme from chick intestine, comprising of an endopeptidase, a carboxypeptidase and a glutamyl aminopeptidase acts in consecutive reactions at neutral pH to hydrolyze pteroylpolyglutamates [17]. These authors contend that earlier techniques depending upon unspecific microbiological growth responses were unreliable in the detection of folyl intermediates formed during the reaction, and suggest that naturally occurring carboxypeptidases may in fact generally represent multienzyme complexes which cleave pteroyl γ-oligoglutamates in consecutive multistep reactions. The present studies however, with purified enzyme preparations and a defined folylpolyglutamyl substrate, employ microbiological growth responses to detect the reaction products after their folate constituents have been chromatographically separated and identified by analytical procedures. Thus the evidence for the sequence of end-products formed, suggesting the successive terminal γ-glutamyl carboxypeptidase activity of the chick hepatic enzyme is unequivocal.

The enzyme has a characteristic double pH optima at pH 4.1 and 5.2. Similar observations have been reported with rat bone marrow enzyme studied earlier in our laboratory [8] and by Tamura et al. [39] for the cabbage enzyme. β-Mercaptoethanol activates the enzyme suggesting that it requires intact thiol groups for activity similar to preparations from green leaves, blood plasma and the bovine liver which are also reportedly activated by thiols [40,41,16]. The thermal stability of the enzyme and its relatively high temperature optimum are evidence of the primitive origin of the protein, consistent with the wide phylogenetic distribution of the enzyme [1].

The relatively high concentrations of various cations required for stimulation of the enzyme suggest an ionic strength effect rather than any specific ion requirement for enzyme. High anion concentrations probably interfere with the polyanion nature of the polyglutamate substrate essential for its binding to the active site at the optimal pH [16]. The potent inhibition by citrate ions at higher concentrations and the autolytic effect promoted by phosphate ions are suggestive of physiological mechanisms that could regulate the enzyme in vivo. Further characterization of the structure and function of the enzyme is the subject of the next paper [21] in this series.

References

1 Stokstad, E.L.R. and Koch, J. (1967) Physiol. Rev. 47, 83—116
2 Bird, O.D., Robbins, M., Vandenbelt, J.M. and Pfiffner, J.J. (1946) J. Biol. Chem. 163, 649—659

3 Bolinder, A., Widoff, E. and Ericson, L.E. (1954) Arkiv. Kemi. 6, 487—502
4 Swendseid, M.E., Bethell, F.H. and Bird, O.D. (1951) Cancer Res. 11, 864—867
5 Hoffbrand, A.V. and Peters, T.J. (1969) Biochim. Biophys. Acta 192, 479—485
6 Baugh, C.M., Stevens, J.C. and Krumdieck, C.L. (1970) Biochim. Biophys. Acta 212, 116—125
7 Landon, M.J. (1972) Int. J. Biochem. 3, 387—388
8 Viswanathan, G. and Noronha, J.M. (1971) Radiat. Res. 42, 141—150
9 Kesavan, V. and Noronha, J.M. (1971) Int. J. Radiat. Biol. 19, 205—214
10 Silink, M. and Rowe, P.B. (1975) Biochim. Biophys. Acta 381, 28—36
11 Saini, P.K. and Rosenberg, I.H. (1974) J. Biol. Chem. 249, 5131—5134
12 Mims, V. and Laskowski, M. (1945) J. Biol. Chem. 160, 493—503
13 Volcani, B.E. and Margalith, P. (1957) J. Bacteriol. 74, 646—655
14 Kazenko, A. and Laskowski, M. (1948) J. Biol. Chem. 173, 217—221
15 Baugh, C.M. and Krumdieck, C. (1969) Lancet 2, 519—521
16 Silink, M., Reddel, R., Bethel, M. and Rowe, P.B. (1975) J. Biol. Chem. 250, 5982—5994
17 Rosenberg, I.H. and Neumann, H. (1974) J. Biol. Chem. 249, 5126—5130
18 Dabrowska, W., Kazenko, A. and Laskowski, M. (1949) Science 110, 95
19 Meister, A. (1973) Science 180, 33—39
20 Price, P.A., Otsuka, A.S., Poser, J.W., Kristaponis, J. and Raman, N. (1976) Proc. Natl. Acad. Sci. U.S. 73, 1447—1451
21 Narasimha Rao, K. and Noronha, J.M. (1977) Biochim. Biophys. Acta 481, 608—616
22 Werkheiser, W.C. and Bartley, W. (1957) Biochem. J. 66, 79—91
23 Potter, V.R. (1957) in Manometric Techniques (Umbreit, W.W., Burris, R.H. and Stauffer, J.F., eds.), 3rd edn., pp. 173—177, Burgess Publishing Co., Minneapolis
24 Swanson, M.A. (1950) J. Biol. Chem. 184, 647—659
25 Gianetto, R. and de Duve, C. (1955) Biochem. J. 59, 433—438
26 Lowry, H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
27 Schertel, M.E., Boehne, J.W. and Libby, D.A. (1965) J. Biol. Chem. 240, 3154—3158
28 Noronha, J.M. and Aboobaker, V.S. (1963) Arch. Biochem. Biophys. 101, 445—447
29 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
30 Smith, I. (1968) in Chromatographic and Electrophoretic Techniques (Smith, I., ed.), 2nd edn., pp. 365—418, Interscience Publishers, Inc., New York
31 Noronha, J.M. and Silverman, M. (1962) J. Biol. Chem. 237, 3299—3302
32 Gawthorne, J.M. and Smith, R.M. (1973) Biochem. J. 136, 295—301
33 Silverman, M., Law, L.W. and Kaufman, B. (1961) J. Biol. Chem. 236, 2530—2533
34 Sumner, J.B. (1944) Science 100, 413—414
35 Soru, E. and Ionescu-Stoian, F. (1965) J. Chromatogr. 17, 538—541
36 Osborne-White, W.S. and Smith, R.M. (1973) Biochem. J. 136, 265—278
37 Chan, C., Shin, Y.S. and Stokstad, E.L.R. (1973) Can. J. Biochem. 51, 1617—1623
38 Scott, Jr., T.A. and Melvin, E.H. (1953) Anal. Biochem. 25, 1656—1666
39 Tamura, T., Buehring, K.U. and Stokstad, E.L.R. (1972) Proc. Soc. Exp. Biol. Med. 141, 1022—1025
40 Iwai, K. (1957) Mem. Res. Inst. Food Sci. Kyoto Univ. 13, 1—9
41 Toennies, G. and Phillips, P.M. (1959) J. Biol. Chem. 234, 2369—2372