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Partial Purification and Some Properties of Pteroylpolyglutamate Hydrolase (Conjugase) from Chicken Pancreas[1] (39612)

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Most of the folates in the tissues of plants and animals occur in the form of poly-γ-glutamyl peptides having from four to seven glutamic acid residues attached in gamma peptide linkage to the glutamyl moiety of folic acid (1-7). These pteroylpolyglutamates do not support the growth of the commonly used assay organisms (*Lactobacillus casei, Streptococcus faecium,* and *Pediococcus cerevisiae*) employed in the microbiological determination of folates (8). To quantitate the folates present in a biological sample, it is therefore necessary to quantitatively cleave the poly-γ-glutamyl chain to form either pteroylmonoglutamates or diglutamates which the assay organisms can efficiently utilize. This is accomplished by prior enzymatic digestion of the sample employing crude preparations of one of a number of pteroyl-poly-γ-glutamyl hydrolases commonly known by the trivial name of "conjugases." The use of different enzyme preparations obtained from a variety of sources (chicken pancreas, hog kidney, human plasma, etc.) having clearly different properties and often containing significant amounts of contaminating folates, is one of the factors that precludes the direct comparison of results of folate assays obtained by different laboratories. As a consequence, there is now considerable confusion regarding the folate content of natural materials and, most importantly, foodstuffs. For these reasons, it seemed desirable to develop a simple procedure for the extraction and partial purification of a conjugase from a readily available source, resulting in a stable folate-free preparation which would permit a better standardization of the techniques of assay of folates. Here we describe a rapid and highly reproducible procedure for the purification of chicken pancreas conjugase yielding a stable preparation in quantity. The preparation has been used to determine molecular weight and other properties of the enzyme.

Materials and methods. Assay of conjugase. Conjugase activity was monitored by the method of Krumdieck and Baugh (9) which measures the release of radioactivity from synthetic pteroyltriglutamate bearing label in the terminal glutamate. The assay conditions used in this report were: 0.5 ml of 0.1 *M* buffer (acetate, pH 4.5, unless otherwise stated); 0.1 ml of 0.001 *M* glutamic acid; 0.5 ml of water; 0.25 ml of 0.1 m*M* pteroyl-γ-glutamyl-γ-glutamyl-([U-^{14}C]glutamic acid) (sp act 0.3 μCi/μmole); and 0.1 ml of enzyme solution. The mixture was incubated for 10 min at 37° and the reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid. The unreacted substrate was removed by adsorption onto charcoal, and the nonadsorbed radioactive glutamic acid liberated by enzyme action was counted in a Beckman LS-250 liquid scintillation counter.

Purification procedure. All operations were performed at 4° unless otherwise stated. Lyophilized chicken pancreas (Difco Laboratories, Detroit, Mich.), in lots of 4 to 10 g, was homogenized for 1 min in approximately 25 vol of 2.5 *M* mercaptoethanol in a top-drive Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was then centrifuged for 10 min at 6000 rpm and the precipitate was discarded. Water was added to the supernate to bring the concentration of mercaptoethanol to 1 *M*, and the solution was centrifuged as previously described to remove the newly formed precipitate. The clear yellow-green supernate so

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obtained was passed through a DEAE-cellulose column (2.5 × 20 cm) previously equilibrated with 200 ml of 0.1 *M* mercaptoethanol. The elution was carried out with 4 column vol of 0.1 *M* mercaptoethanol, or until the eluate was free of conjugase activity. The conjugase was then further purified by adsorption and elution from aged alumina C-γ-gel (5% solids content; Sigma Chemical Company). Alumina C-γ-gel and 1 *M* tris-(hydroxymethyl)-aminomethane buffer (pH 6.5) in 0.1 *M* mercaptoethanol were added to the eluate in a ratio of 1:6:50, respectively, and the mixture was stirred for 15 min before centrifugation for 10 min at 6000 rpm. The supernatant fraction was discarded, and the precipitate was stirred in 20 ml of 0.1 *M* phosphate buffer at pH 6.5 for 15 min and centrifuged for 10 min at 6000 rpm. The last step was repeated and the supernates were combined. To produce a stable preparation, the supernate was dialyzed overnight against 20 vol of 2 *M* sucrose; the nondialyzable remnant was then removed from the cellophane casing by rinsing with 15 ml of distilled water, lyophilized, and vacuum-sealed.

Molecular weight estimation. The molecular weight of the enzyme was estimated by Sephadex G-75 gel filtration. The column (2.5 × 34 cm) was equilibrated and eluted with 0.01 *M* Tris buffer (pH 6.5) in 0.02% sodium azide and 0.1 *M* mercaptoethanol, and standardized with ribonuclease A (mol wt 13,700), chymotrypsinogen A (mol wt 25,000), and ovalbumin (mol wt 45,000). The molecular weights of the calibration proteins and the procedures are those given in Pharmacia Fine Chemicals Calibration Kit (Piscataway, New Jersey).

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (10) using bovine serum albumin as protein standard.

Paper chromatography. Ascending paper chromatography in 0.2 *M* ammonium acetate buffer (pH 5.8) was used to identify the end product of conjugase action on synthetic pteroylheptaglutamate. Standards of poly-γ-glutamyl derivatives of folic acid (11) were run simultaneously. The folic acid derivatives were detected by uv light absorption.

Results. The summary of purification steps for chicken pancreas conjugase is presented in Table I. The final purification was approximately 50-fold over the activity in the initial mercaptoethanol homogenate, and the yield was 53%. No loss of conjugase activity could be detected after 5 months of storage at −20° of the vacuum-sealed lyophilized preparation. In contrast, the enzyme activity of the DEAE-cellulose eluate decreases gradually with about 50% loss within 1 week at 4°. The alumina C-γ-gel eluate prior to dialysis is even less stable. No detectable folate activity was found in the conjugase preparation when assayed with *L. casei* as the assay organism.

The end product of the enzyme action upon synthetic pteroylheptaglutamate was identified as pteroyldiglutamate by paper chromatography with authentic reference standards.

The elution profile of conjugase activity from Sephadex G-75 shows the presence of two peaks (Fig. 1). The molecular weights of the two components correspond approximately to 50,000 and 25,000, respectively.

The two activity peaks were pooled separately and rechromatographed on a Sephadex G-75 column. The first peak (tubes 30–

TABLE I. SUMMARY OF CONJUGASE PURIFICATION STEPS.[a]

Step	Volume (ml)	Protein (mg)	Total activity (cpm)	Specific activity (cpm/mg protein)	Yield (%)
Mercaptoethanol homogenate	50	2,800	330,000	118	100
Mercaptoethanol extract	83	246	353,320	1,436	107
DEAE-cellulose eluate	250	180	312,500	1,736	95
Alumina C-γ-gel eluate	30	34.8	175,500	5,043	53

[a] This is a typical result with 4 g of Difco chicken pancreas, and all the purification steps are highly reproducible.

45) eluted again as a single molecular species in the same position as before. The second peak on the other hand (tubes 46–65) resulted in two peaks of activity essentially identical to the pattern obtained originally (Fig. 1).

The partially purified conjugase also exhibited these two peaks on Sephadex G-75 gel filtration when mercaptoethanol was omitted from both the purification procedure and the elution solvent.

The optimum temperature for conjugase activity from chicken pancreas was between 35 and 50° at pH 8 (shown in Table II). In the pH range 4.0–10.0 at 37°, the enzyme activity increases with pH up to 8.5 and then decreases (Table III).

Discussion. The purification procedure described reproducibly yields a stable preparation of chicken pancreas conjugase which is free of folate and readily obtained in sufficient quantities for use in standardized microbiological assays of folates in natural materials.

The present study confirms the observations of Kazenko and Laskowski (12) and of Jagerstad *et al.* (13) that the product of chicken pancrease conjugase digestion is pteroyldiglutamate. This is also the end product of conjugase purified from chicken intestinal mucosa by Saini and Rosenberg (14).

Our results indicate that the folate conjugase of chicken pancreas consists of two subunits each with a molecular weight of approximately 25,000. Disulfide bridge formation is probably not essential for the formation of the 50,000-molecular weight aggregate, since it is formed in either the presence or absence of mercaptoethanol.

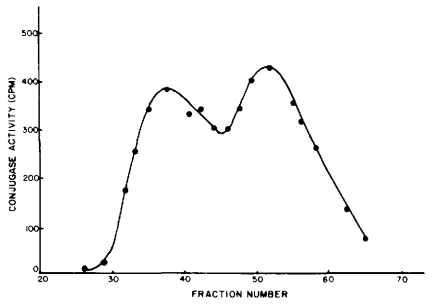

FIG. 1. Elution profile of conjugase activity on Sephadex G-75 (2.5 × 34 cm) with 0.01 Tris buffer (pH 6.5) in 0.02% NaN_3 and 0.1 *M* mercaptoethanol as eluant. Fraction size, 2.0 ml.

TABLE II. CONJUGASE ACTIVITY AS A FUNCTION OF TEMPERATURE IN 0.1 *M* TRIS BUFFER, pH 8.

Temperature (°)	Conjugase activity (cpm)
20	344
35	708
40	722
45	739
50	757
55	696
60	472

TABLE III. CONJUGASE ACTIVITY AS A FUNCTION OF pH AT 37°.

Buffer	pH	Conjugase activity (cpm)
0.1 *M* Na acetate		
	4.0	107
	4.5	136
	5.0	179
	5.5	241
0.1 *M* MES[a]		
	6.0	305
	6.5	389
	7.0	581
0.1 *M* Tris[b]		
	7.5	633
	8.0	706
	8.5	769
	9.0	738
0.1 *M* Borax-NaOH		
	9.3	709
	9.7	18
	10.1	13

[a] (2-[*N*-Morpholine])ethanesulfonic acid.
[b] (Hydroxymethyl)-aminomethane.

Jagerstad *et al.* (13) have also reported that chick pancreas conjugase has a molecular weight of 52,000 by comparison with known standards on gel chromatography, but have observed only a single peak of activity. Although their starting material was the same as that used in the present study, no preliminary purification was used and the analytical procedures differed in several respects. Thus different gel and buffer solutions were used in the two investigations, and different methods were employed to detect enzyme activity. Jagerstad *et al.* (13) used microbiological assay to detect the ability of fractions to convert yeast pteroylpolyglutamate to a form capable of supporting the growth of *L. casei*. It seems

possible that these conditions might not have favored the dissociation of the enzyme or the detection of enzyme activity in subunit fractions.

The enzyme from chicken intestinal mucosa purified by affinity chromatography by Saini and Rosenberg (14) has a molecular weight of 80,000 and no subunits.

The optimum pH for chicken pancreas conjugase is appreciably higher than the optimum pH reported for conjugases from most other tissues (1, 15–17). The latter are primarily lysosomal enzymes with characteristically acidic pH optima. Although the intracellular distribution of chicken pancreas conjugases has not been studied, its high pH optimum argues against its being a lysosomal hydrolase.

Summary. A simple and rapid procedure for the purification of pteroylpolyglutamate hydrolase (conjugase) from chicken pancreas for the purpose of standardization of microbiological assays of folates has been developed. It yields a stable folate-free preparation in quantity. The purification steps included extraction of conjugase from crude lyophilized chicken pancreas in mercaptoethanol followed by DEAE-cellulose chromatography. The enzyme was further purified by absorption to alumina-C-γ-gel from which it was eluted with phosphate buffer, pH 6.5. The enzyme was concentrated by dialysis against 20 vol of 2 *M* sucrose, lyophilized, and vacuum-sealed. The purified conjugase exhibited two peaks on Sephadex G-75 chromatography corresponding to molecular weights of 50,000 and 25,000, respectively. The optimum temperature for the conjugase was between 35 and 50°, and the optimum pH was 8.5.

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