PURIFICATION OF MOUSE PEPSINOGENS BY PEPSTATIN-AFFINITY CHROMATOGRAPHY

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1. Introduction

Pepsin and pepsinogen, especially those of hog and human, are the most extensively studied acid proteases and the primary structure of hog pepsin has been established [1]. However, although mice and rats are widely used experimental animals, little is known about their pepsin and pepsinogen.

We purified two forms of mouse pepsinogen (PgI and PgII) to homogeneity, using pepstatin–Sepharose 4B affinity chromatography, gel filtration and Sephadex G-100 and DEAE–Sepharose CL6B column chromatography. This is the first report on the purification of mouse pepsinogens. Moreover, this result provides the evidence that pepsinogens per se have an ability to bind to pepstatin, a specific acid protease inhibitor [2,3].

2. Materials and methods

Pepstatin, antipain and leupeptin, which are protease inhibitors purified from culture medium of *Actinomycetes*, were generous gifts from Dr H. Umezawa, Inst. Microbial Chem., Tokyo. Aminohexyl–Sepharose 4B, Sephadex G-100 and DEAE–Sepharose CL6B were products of Pharmacia Fine Chemicals, Uppsala. Bovine hemoglobin and crystallized hog pepsin were purchased from Sigma, St Louis, MO. All other chemicals used were commercial products of reagent grade.

Pepstatin–Sepharose 4B was prepared as in [4]. The swollen gel could bind 3 mg crystallized hog pepsin/ml, at pH 2.2.

Protease activity was determined, at pH 2.9, as in [5] with the slight modification in [6]. One unit of activity was defined as the amount which caused an increase of 1 A_{280}/min under the above conditions. Milk clotting activity was measured as in [7]. Protein was determined as in [8] using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis was carried out using 7.5% polyacrylamide gel and 50 mM Tris–acetate buffer (pH 8.2) or 50 mM acetate buffer (pH 5.7). Enzyme on the gel was located as in [9]. Protein was stained with 0.2% Coomassie brilliant blue R-250. SDS–polyacrylamide gel electrophoresis was performed as in [10].

3. Results

The purification steps are summarized in table 1. Stomachs were excised from about 10-week old male BDF_1 mice obtained from Shizuoka Nokyo, Shizuoka. Mucosal tissue of the glandular stomach was scraped off with a razor balde and homogenized with a Potter-Elvehjem homogenizer in 9 vol. 0.01 M sodium phos-

Table 1
Purification of mouse pepsinogens

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg prot.)	Recovery (%)
Supernatant, 105 000 × *g*	1548	137.8	11.2	100
Supernatant, pH 3.9	1470	78.0	18.8	95.0
Pepstatin–Sepharose 4B	1500	17.4	86.2	96.0
Sephadex G-100	800	6.7	119	51.7
1st DEAE–Sepharose CL6B 1[a]	386	3.3	117	24.9
2[a]	357	2.7	131	23.1
2nd DEAE–Sepharose CL6B PgI	172	1.45	118	11.1
PgII	196	1.40	140	9.0

[a] 1 and 2 are pools of fractions containing the slower and faster eluting peaks of activity, respectively

phate buffer (pH 7.0) containing 50 μg/ml antipain and leupeptin. All purification procedures were performed at 4°C unless otherwise stated. The homogenate was stirred for 1 h and then centrifuged at 105 000 × *g* for 60 min. Almost all protease activity was recovered in the supernatant, and this fraction contained at least 5 bands of activity of acid protease on gel electrophoresis but approx. 90% activity belonged to PgI and PgII.

The supernatant was mixed with equal vol. 0.1 M citrate phosphate buffer (pH 3.8) containing 0.5 M NaCl (buffer A), to give final pH 3.9. Insoluble material was removed by centrifugation at 27 000 × *g* for 10 min. The supernatant was mixed with 1/4 vol. pepstatin–Sepharose 4B, which had been equilibrated with buffer A. This mixture was overlaid on a pepstatin–Sepharose 4B column (1.6 × 3 cm) which had also been equilibrated with the same buffer. The column was washed successively with 5 column vol. buffer A and 0.5 M NaCl, then the acid protease was eluted with 0.01 M boric acid–borax buffer (pH 8.5) containing 0.5 M NaCl. Fractions containing acid protease activity were combined and mixed with solid ammonium sulfate to give 65% saturation. The precipitate was dissolved in a small vol. 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and 20% glycerol (buffer B). The solution was then subjected to gel filtration on a Sephadex G-100 column (2.6 × 90 cm), eluted with buffer B. Two peaks of acid protease activity were obtained in this step. Fractions of eluate in the major peak of activity which contained PgI and PgII were combined and chromatographed on a DEAE–Sepharose CL6B column (1.4 × 20 cm) which had been equilibrated with buffer B. The column was eluted with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.165 M NaCl and 20% glycerol. One broad peak of activity was obtained in this step. However, the faster-eluting part of the peak contained mainly PgII and the slower one PgI. The broad peak of activity was divided into two parts and pooled. These two pools of acid protease activity were separately subjected to rechromatography on DEAE–Sepharose CL6B column under the same conditions.

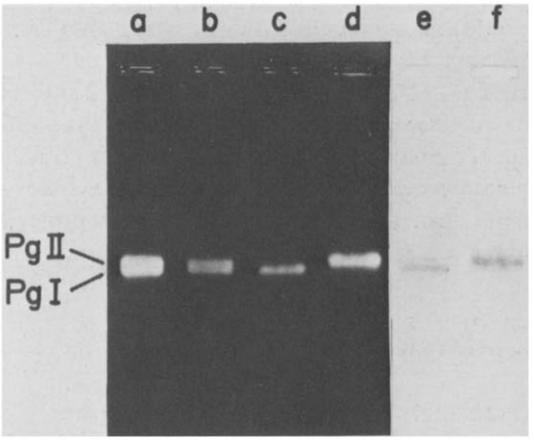

Fig.1. Gel electrophoretic patterns of activity and protein at various purification steps. (a) Activity of 105 000 × *g* supernatant; (b) activity of pepstatin–Sepharose 4B fraction; (c,d) activities of purified PgI and PgII, respectively; (e,f) protein of purified PgI and PgII, respectively. 7.5% polyacrylamide gel and 50 mM Tris–acetate buffer (pH 8.2) were used.

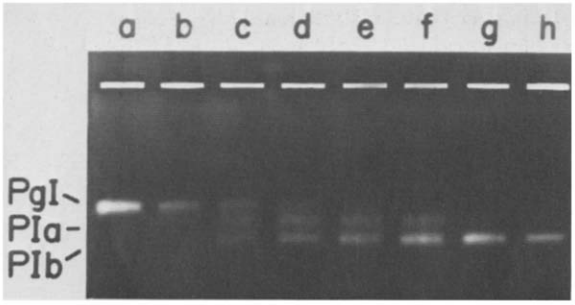

Fig.2. Gel electrophoretic patterns of PgI after acid treatment, at pH 2.2 and 25°C, for various times. The raction was stopped by mixing an aliquot of the reaction mixture with 5 vol. 50 mM sodium acetate buffer (pH 5.7). Gel electrophoresis was performed using 7.5% polyacrylamide gel and sodium acetate buffer (pH 5.7). After electrophoresis, activities were visualized at pH 3.0. (a–h) Activation for 0 s, 10 s, 20 s, 30 s, 1 min, 5 min, 20 min and 40 min, respectively.

Each preparation of PgI and PgII showed single band of activity and protein on polyacrylamide gel electrophoresis (fig.1). The molecular weight of both pepsinogens were determined to be 41 000 on SDS–gel electrophoresis (data not shown).

A solution, 800 μg/ml purified PgI was activated at pH 2.2, 25°C, and subjected to polyacrylamide gel electrophoresis at pH 5.7. As shown in fig.2, PgI gave two bands of active pepsins (PIa and PIb). At an early stage of activation, PIa was predominant and in 40 min activation only PIb was observed. This was also the case with PgII (data not shown). Two pepsins derived from PgII were called PIIa and PIIb. The molecular weights of PIa and PIIa were calculated to be 38 000 and that of PIb and PIIb to be 36 000 by SDS–gel electrophoresis.

After activation, at pH 2.2, PgI and PgII showed milk-clotting activity at pH 5.3. PgI, 0.5 μg, showed this activity in 60 s at 37°C and 0.5 μg PgII in 67 s. However, without activation, they showed no activity.

4. Discussion

The present work showed that a pepstatin Sepharose 4B column was effective for isolating pepsinogens from the gastric mucosa of mice. The two enzymes that were purified to homogeneity were regarded as pepsinogens for the following reasons:

(1) These two enzymes comprised approx. 90% total acid protease activity of mouse gastric mucosa crude extract.

(2) Without activation, PgI and PgII did not show milk-clotting activity, at pH 5.3, but after activation they did.

(3) On acid treatment, between pH 2 and pH 3, PgI and PgII decreased in molecular size within 5 min at 25°C but retained their potential peptic activities; whereas at pH 4, this reaction took place very slowly and above pH 5, did not take place at all (data not shown).

At an early stage of activation, two forms of pepsin were observed. However, the smaller form of pepsin became predominant with time of activation. These observations suggest that PIa and PIIa are active intermediates in the activation of mouse pepsinogens, as suggested recently in the porcine pepsinogens [11].

The present work also provides the evidence that pepsinogen can bind to pepstatin without cleavage of activation peptide and agrees well with [12].

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