ISOLATION AND SOME PROPERTIES OF CHICKEN PEPSIN

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A novel method of isolation of chicken pepsins by chromatography on Sepharose 4B with a covalently attached mercury derivative is described. The chief advantages of this isolation procedure are a 6-fold increase of the specific activity of the preparation in one operation and minimum losses of active material. The preparation obtained was resolved into 4 individual forms by chromatography on DEAE-cellulose. All forms showed the presence of N-terminal serine. They have the same specificity when tested with the B-chain of oxidized insulin as substrate; this specificity differs from that of hog pepsin.

The molecule of chicken pepsin and its zymogen, unlike other animal pepsins so far known, has a free thiol group[1,2]. This thiol group is obviously located on the surface of the molecule and in native unmodified pepsin it can be not only titrated but also chemically modified[3]. We considered therefore worthwhile to use the presence of this free SH-group for the isolation of chicken pepsin by affinity chromatography. In this paper we report on the isolation of chicken pepsin on a mercurial Sepharose column and on the resolution of its individual forms and their characterization.

EXPERIMENTAL

Material. Chicken forestomachs (proventriculi) were obtained from young chickens not later than 1 h after the slaughter. Adhering fat was removed, the organs were washed with cold physiological saline and frozen to −20°C. Hog pepsin was a 2× crystallized and lyophilized preparation of Worthington, U.S.A. Insulin was a product of Léčiva, Prague. The B-chain of oxidized insulin was prepared electrophoretically at 1500 V in this laboratory. Sepharose 4B and Sephadex G-75 were products of Pharmacia, Sweden. DEAE-cellulose was from Calbiochem, U.S.A. *p*-Aminophenylmercuric acetate was from Aldrich, Belgium.

Proteolytic activity assay. The pepsin solution (100 µl) was added to 1 ml of 2% solution of acid-denatured hemoglobin (pH 2). After 10 min hydrolysis at 39°C, 2 ml of 5% solution of trichloroacetic acid was added. The mixture was filtered and the A_{280nm} of the filtrate was determined. One unit of enzyme activity was defined as that amount of enzyme which increased the absorbance (ΔA_{280nm}) by 1·0 in 1 min. Specific activity defines the number of enzymic units in 1 mg of protein.

Crude pepsin was prepared from chicken forestomachs by the procedure of Bohak[1]. A total of 24 g of crude pepsin was obtained from 1 kg of tissue.

Mercurial Sepharose column. p-Aminophenylmercuric acetate was coupled to cyanogen bromide activated Sepharose[4]. One ml of our preparation of mercurial Sepharose was capable of binding 2 μmol of 2-nitro-5-thiobenzoic acid[4]. This Sepharose preparation was regenerated as necessary by 6M guanidine chloride solution at pH 6 and could then be used for a considerably higher number of runs than usual.

The preparation of the dansyl derivatives of pepsin and their hydrolysis were carried out as described by Gray.[5] The DNS-amino acids were identified by thin-layer chromatography[6,7].

The digestion of the B-chain of oxidized insulin with pepsin was carried out in 0·01M-HCl, 2 h at 37°C; the enzyme substrate ratio was 1 : 100 (w/w). The digests were examined by the method of peptide maps. A 500 μg sample of the digest was applied to Whatman paper 3 MM. Electrophoresis was used in the first direction and chromatography in the second direction. The peptides were stained with 0·2% solution of ninhydrin in acetone.

Disc electrophoresis was run in 7·5% polyacrylamide gel in Tris-glycine buffer, pH 8·3.

RESULTS AND DISCUSSION

Crude active chicken pepsin[1] was used to start with. Its chromatography on a mercurial Sepharose column, equilibrated with 0·05M acetate, pH 5, containing 0·2M-KCl, 0·001M-EDTA, and 0·5% of n-butanol, is shown in Fig. 1. After the elution of the first peak, which was lacking proteolytic activity, the proteins attached to the column were displaced by 0·02M 2-mercaptoethanol in the acetate buffer. The yield in terms of activity units was about 90% and the specific activity increased approximately 6-times (Table I). We preferred a thiol group-containing reagent to mercury(II) chloride for the displacement of chicken pepsin since the blocking of the SH-group by mercury(II) chloride would lead to the inactivation of the enzyme[1]. Because all

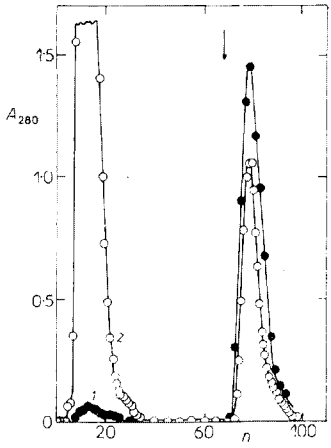

FIG. 1

Chromatography of Chicken Pepsin on Mercurial Sepharose 4B Column

Crude chicken pepsin (500 mg) was applied onto a 2·5 × 16 cm column of mercurial Sepharose, equilibrated with 0·05M acetate, pH 5, containing 0·2M-KCl, 0·001M-EDTA, and 0·5% of n-butanol. Flow rate 90 ml/h. Attached pepsin was eluted by 0·02M 2-mercaptoethanol in the acetate buffer, pH 5. *1* absorbance at 280 nm; *2* proteolytic activity; *n*, tube number.

TABLE I
Isolation and Separation of Chicken Pepsin

Isolation procedure	Specific activity units/mg prot.	Yield %
Crude pepsin	3·0	100
Mercurial Sepharose	20·0	92
DEAE-cellulose II	10	3·3
DEAE-cellulose III	10	3·8
DEAE-cellulose IV	18·0	10
DEAE-cellulose V	25·0	30

the compounds bearing a free thiol group are attached to mercurial Sepharose, the chromatographic procedure described by us here could be also used for the preparation of chicken pepsinogen.

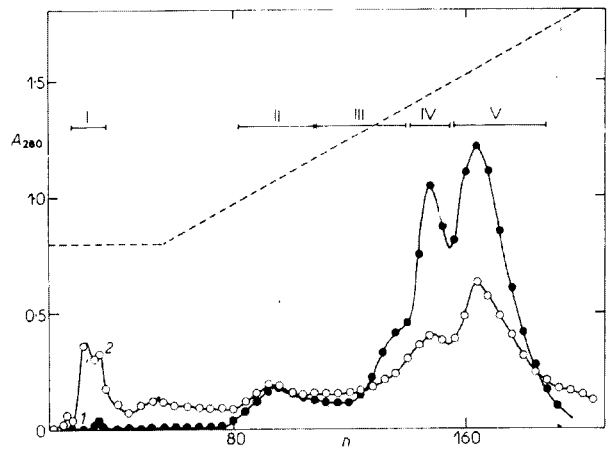

FIG. 2
Chromatography of Chicken Pepsin on DEAE-Cellulose
Column 1·5 × 25 cm, flow rate 9 ml/h. Initial buffer 0·02M phosphate, pH 6·9, 0·2M in NaCl. As soon as the absorbance dropped below 0·100, the column was eluted by a linear gradient developed by passing 300 ml of 0·02M phosphate, pH 6·9, 0·4M in NaCl, in 300 ml of the initial buffer. After emergence of fraction No 230 the column was eluted by 0·02M phosphate, pH 6·9, 0·6M in NaCl. *1* absorbance at 280 nm; *2* proteolytic activity; I—V pooled fractions; ——— molarity of NaCl (0·2—0·4M); *n*, tube number.

When the active fraction from the mercurial Sepharose column is subjected to disc electrophoresis it shows the presence of two main zones and at least 4 minor zones of lower mobility. We chromatographed therefore this fraction on DEAE-cellulose. As shown in Fig. 2, the proteolytic activity is distributed in all fractions. Only traces of activity are in the first peak, the main bulk of activity is in the fraction bearing the most negative charge. Fraction V was subsequently chromatographed on a DEAE--cellulose column in 0·02M phosphate buffer, eluted by a linear gradient of 0·27M to

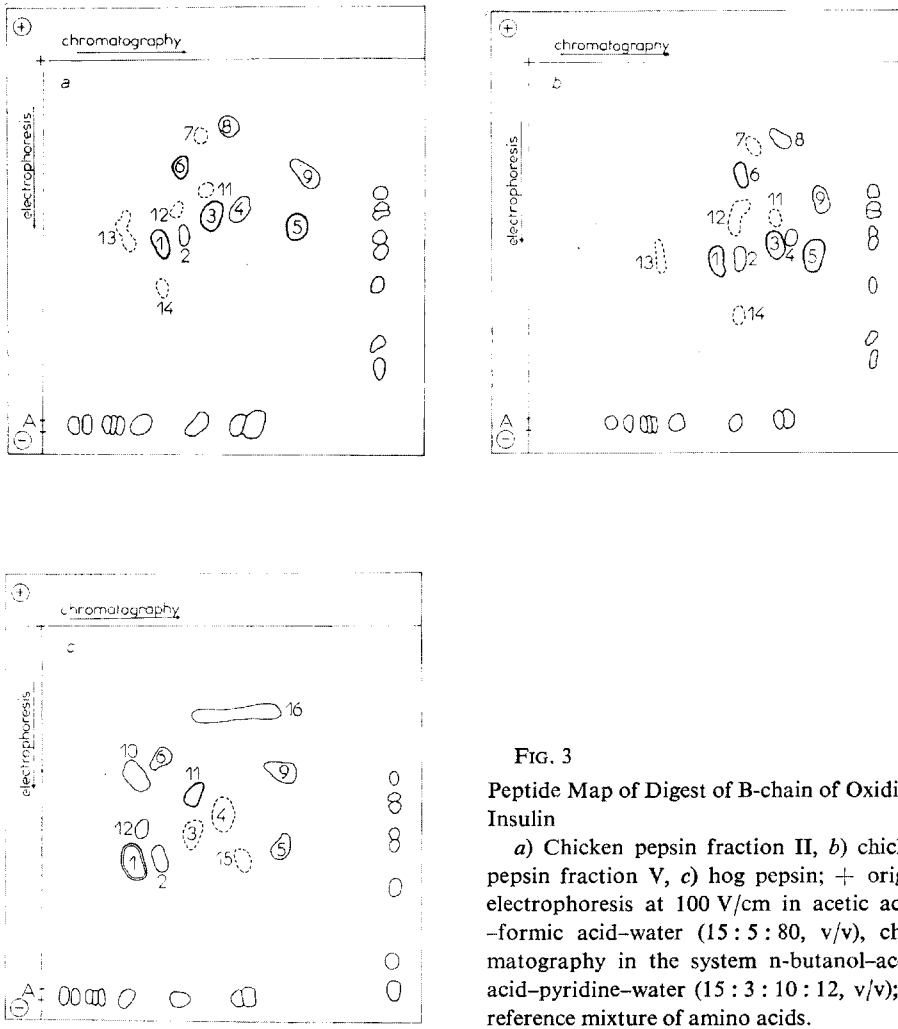

FIG. 3

Peptide Map of Digest of B-chain of Oxidized Insulin

a) Chicken pepsin fraction II, *b*) chicken pepsin fraction V, *c*) hog pepsin; + origin; electrophoresis at 100 V/cm in acetic acid–formic acid–water (15 : 5 : 80, v/v), chromatography in the system n-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 12, v/v); A, reference mixture of amino acids.

0·37M-NaCl. After this rechromatography, it was contaminated only to a negligible degree by fraction IV. Appearingly the relative quantity of the individual forms of chicken pepsin decreases with the decreasing negative charge. Hence, the most acidic fraction V of highest specific activity contains approximately 60%, fraction IV 20%, fraction III 8%, fraction II 8% of pepsin activity (Table I). The fact that all forms of chicken pepsin are attached to mercurial Sepharose shows that they all contain a free thiol group in their molecule. All forms of chicken pepsin, isolated by us, show the presence of N-terminal serine. The same reports for his single chicken pepsin Bohak[8]. By contrast, Green and Llewellin determined N-terminal threonine in chicken pepsin 4 (ref.[9]) and report serine for its zymogen. The existence of multiple forms of chicken pepsinogen was demonstrated by several authors earlier. Thus, *e.g.* Levčuk and Orechovich[10] described 3 pepsinogens, Donta and Van Vukanis[2] also 3, Green and Llewellin[9] 5 forms of pepsinogen. We find at least 4 forms of active chicken pepsin in our experiments.

We studied the enzymatic activity of the individual forms of chicken pepsin toward the B-chain of oxidized insulin. Peptide maps of the digests of this substrate by fractions of chicken pepsin obtained by us by chromatography on DEAE-cellulose are shown in Fig. 3. The distribution of peptides in the digests obtained with fraction II (least acidic) and fraction V (most acidic) as well as with fractions III and IV, is identical. This leads us to assume that all the forms isolated by us show the same specificity toward this substrate and that they can therefore be named isozymes. On the other hand, when we compare the peptide maps of the digests of this substrate by hog and chicken pepsin, we observe some differences. As shown in Fig. *3a*, *c*, some peptides (No 1, 5, 6, 9) are common to both maps, others differ in relative intensity (No 2, 3, 4, 11, 12), still others are specific either for chicken pepsin or for hog pepsin only (No 7, 8, 10). Our findings are in agreement with the results of similar experiments reported by Levčuk and Orechovich[11], who also studied the digestion of the B-chain of oxidized insulin by hog and chicken pepsin. Hog and chicken pepsin differ in amino-acid composition; we may therefore assume that they also differ in the composition and perhaps also conformation of their binding sites and that these differences underlie the different enzymatic specificity of the two enzymes.

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