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Pepsin Treatment of Avian Skin Collagen

EFFECTS ON SOLUBILITY, SUBUNIT COMPOSITION AND AGGREGATION PROPERTIES

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1. Collagen was extracted from chick skin with dilute acetic acid followed by dilute acetic acid containing pepsin. 2. The solubilized collagens were purified and portions subjected to further digestion by pepsin. 3. This treatment decreased the aldehyde content but contamination by hexosamine was not diminished. 4. Pepsin treatment converted practically all the acid-soluble collagen into monomeric subunits (α-chains), but the pepsin-solubilized material retained a significant amount of higher subunits (β- and γ-chains). 5. Treatment lowered the rate of fibrillogenesis by acid-soluble collagen, but was without effect on pepsin-solubilized collagen.

The tropocollagen molecule consists of three polypeptide chains, each of molecular weight approx. 95000, extending the whole length of the molecule (Lewis & Piez, 1964*a*; Piez, 1965; Kang *et al.*, 1966). In collagens from several sources the amino acid composition of one of the chains (the α2-chain) differs significantly from that of the other two (the α1-chains) (Piez *et al.*, 1963; Lewis & Piez, 1964*b*; Bornstein *et al.*, 1964).

In its native state tropocollagen is resistant to attack by most proteases with the exception of a group of enzymes called collagenases, found in certain bacteria and in many (probably all) vertebrates. However, careful studies by Hodge *et al.* (1960) and later by Rubin *et al.* (1963) and Steven (1965, 1966) have demonstrated that, although the gross physical characteristics of the molecule remain unchanged, subtle alterations occur when tropocollagen is treated with proteolytic enzymes such as pepsin or trypsin. Prominent among these changes are a slight fall in viscosity, inhibition or diminution of the capacity to form native-type fibrils *in vitro*, conversion of β-subunits (dimers) into α-subunits (monomers) and the release of small quantities of peptide material with an amino acid composition that differs from that of collagen as a whole, in that it contains relatively little glycine, proline or hydroxyproline and is relatively rich in tyrosine, phenylalanine, aspartic acid and glutamic acid. These findings are thought to demonstrate the presence in tropocollagen of small amino acid sequences that do not comply with the accepted triple-helical structure for tropocollagen (Ramachandran, 1967). They are known as 'telopeptides' and are believed to be essential for end-to-end aggregation of tropocollagen molecules, a step that is necessary for fibrillogenesis (Hodge *et al.*, 1960). It is now known that the lysine residues involved in intramolecular covalent cross-linking of α-chains are located in these telopeptide regions at the *N*-terminus of the α-chains (for a review see Piez, 1968).

Fujimoto (1968) found that, as a preliminary to purification from the muscle layer of *Ascaris lumbricoides* and pig kidney, collagen could be solubilized by pepsin treatment, and we have since applied the same technique to solubilization of avian intramuscular collagen (Bannister & Burns, 1972). We found that tropocollagen from intramuscular sources was more highly cross-linked than similarly treated material from skin. Although solubilization results from digestion of non-collagenous protein, it is also the result of cleavage of telopeptides containing covalent cross-links, and hence comparisons between collagens from different sources could not be made with precision.

These observations have prompted further investigation of the effects of pepsin, and the present paper deals with solubilization of avian skin collagen by the enzyme and its action on some properties of the purified protein.

Experimental

Solubilization and purification of collagen

The source of material for all experiments was the skin of a strain of domestic fowl (derived from commercial broilers). The birds were all female and aged between 12 and 18 weeks. Recently synthesized collagen was extracted with acetic acid and most of the remaining (older) protein was solubilized by treatment with pepsin (EC 3.4.4.1). Details of the procedure are as follows. The plucked skins from freshly killed birds were cleaned of adhering fat and muscle and minced, with ice, in a hand-operated mincer. The

material was defatted by two extractions with chloroform–methanol (2:1, v/v), washed thoroughly with tap water and distilled water and extracted twice with 0.2M-Na_2HPO_4 at room temperature. All subsequent operations were performed at 0–4°C. Acid-soluble collagen was obtained by extracting the defatted minced skin three times (for 2–3 days in each case) with 3% (v/v) acetic acid. The remaining material was then resuspended in 3% (v/v) acetic acid and digested for 2–3 days with approx. 5mg of pepsin/ml.

The collagens solubilized by both procedures were purified as follows. The extracts were clarified by centrifugation for 1h at approx. 33000*g* and the supernatant was dialysed against aq. NaCl so that the final concentration was 7% (w/v). The precipitated collagen was collected by centrifugation, redissolved in 3% (v/v) acetic acid and centrifuged for 1h at approx. 77000*g*. The whole of this procedure was repeated and the protein was precipitated a third time by dialysis against 0.02M-Na_2HPO_4. After dissolving in, and dialysis against, 3% (v/v) acetic acid the collagen was centrifuged for 1.5h at 109000*g*. The supernatant containing the purified protein was stored, either in solution in a refrigerator or, more often, was freeze-dried and stored in a desiccator.

Pepsin treatment of soluble collagens

Portions of acid-soluble and pepsin-solubilized collagens were adjusted to 1.0mg/ml in 3% (v/v) acetic acid and treated with crystalline pepsin (batch no. 52541; Koch–Light Laboratories, Colnbrook, Bucks., U.K.) (concn. 0.1mg/ml) for periods of between 3 and 14 days at 4°C. After digestion, pepsin was removed by one salt precipitation and one Na_2HPO_4 precipitation, as described above. Thus four types of collagen were available for study: acid-soluble (A), acid-soluble pepsin-digested (Ap), pepsin-solubilized (P) and pepsin-solubilized pepsin-digested (Pp). Unless otherwise stated, collagen Pp had been treated for 14 days.

Hexosamine and aldehyde contents of collagen preparations

The amount of hexosamine present is considered to be a measure of contamination of collagen preparations by the mucopolysaccharide constituents of connective tissue. Accordingly measurements were made by the Elson–Morgan procedure as described by Davidson (1966), with glucosamine as standard. The aldehyde content was assayed by the method of Paz *et al.* (1965), with acetaldehyde as standard.

Determination of subunit composition

The percentage of α-, β- and γ-chains in denatured collagen solutions was determined by polyacrylamide-gel electrophoresis and densitometry as described previously (Bannister & Burns, 1972).

Rate of fibril formation

The rate at which native-type fibrils can be formed from collagen solutions may be used as a measure of the aggregation properties of the tropocollagen molecule. The procedure was as follows. Collagen was dissolved in 3% (v/v) acetic acid at 0°C and the pH carefully raised to 7.2 by adding 2M- and 0.01M-NaOH using a Pye model 291 pH-meter. When the required pH was achieved, the volume was adjusted with water to give a collagen concentration of 0.1%. Fibrillogenesis was monitored continuously at 400nm in a Unicam SP.800 spectrophotometer. The process was initiated by transferring solution from an ice bath to a cuvette maintained at 38°C.

Results and Discussion

Effects of pepsin treatment on hexosamine and aldehyde content

Hexosamine. Contamination by hexosamine was low and similar in all preparations (Table 1). Thus

Table 1. *Hexosamine and aldehyde contents of collagen preparations*

Soluble collagens were prepared and treated with pepsin as described in the Experimental section. The collagen preparations are classified as follows: A, acid-soluble; Ap, acid-soluble, pepsin-treated (3 days); P, pepsin-solubilized; Pp, pepsin-solubilized, pepsin-treated (14 days). The contents were calculated by assuming a molecular weight of 285000 for tropocollagen.

Collagen preparation	Hexosamine content (μmol/μmol of tropocollagen)	Aldehyde content (μmol/μmol of tropocollagen)
A	0.99	0.78
Ap	0.86	0.44
P	0.96	0.37
Pp	1.16	0.24

pepsin treatment is probably without effect in the further purification of already highly purified collagen.

Aldehyde. Table 1 also gives the aldehyde contents of the collagen preparations. The findings are somewhat different from those of Deshmukh & Nimni (1971), in that chick skin acid-soluble collagen contained about one-third as much aldehyde as did neutral-salt-soluble collagen from rat skin. However, this is not surprising, in view of the biologically older nature of acid-soluble collagen and the differing methods employed in purification. Pepsin-solubilized collagen contained about one-half as much as the acid-soluble material, again, presumably, reflecting its greater biological maturity and also some loss of telopeptides due to action of the enzyme. When treated with pepsin, both preparations (Ap and Pp) sustained further decreases in aldehyde content, in conformity with the location of this group in the telopeptide region.

Pepsin treatment

The subunit composition was expressed as the percentage of α-chains (monomers), β-chains (dimers) and γ-chains (trimers), although this last group probably contained higher-molecular-weight subunits as well. The compositions of collagens A and P are given as percentages (measured at zero time) in Fig. 1, which shows that, although there is no great difference in the content of α-chains, collagen P is very much richer (about fourfold) in γ-chains. The digestion of

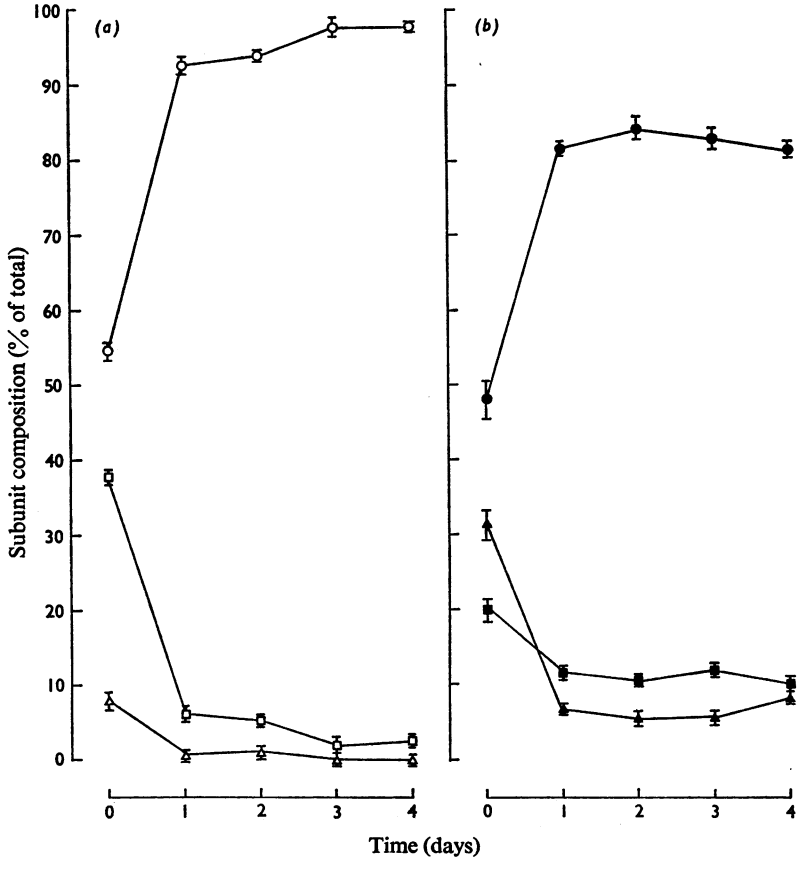

Fig. 1. *Effects of pepsin digestion on the subunit composition of two collagen preparations*

Collagen A (○, □ and △) and collagen P (●, ■ and ▲) were prepared as described in the Experimental section. For details of pepsin treatment and electrophoretic analysis see the text. The results are given as the mean (±S.E.M.) percentages of four to six electrophoretic analyses. ○ and ●, α-Chains; □ and ■, β-chains; △ and ▲, γ-chains.

these two collagens by pepsin was studied by sampling at 24h intervals and examining the urea-denatured material by gel electrophoresis. In this experiment pepsin was not removed by repurification because it did not interfere with separation of the subunit classes. The results of treatment over a 4-day period are shown in Fig. 1. Collagen A was almost completely converted into α-chains with little β- and no detectable γ-chains remaining. In contrast, collagen P gave rise to fewer α-subunits than did collagen A, and significant quantities of β- and γ-chains survived pepsin treatment. This observation suggests that there are covalent cross-links present in collagen P that resist pepsin attack, and confirms a similar conclusion reached by Steven (1966) with bovine and human collagens.

Rate of fibril formation

Gross & Kirk (1958) and Bensusan and co-workers (Bensusan, 1960; Bensusan & Scanu, 1960) studied the effects of a variety of substances on the rate at

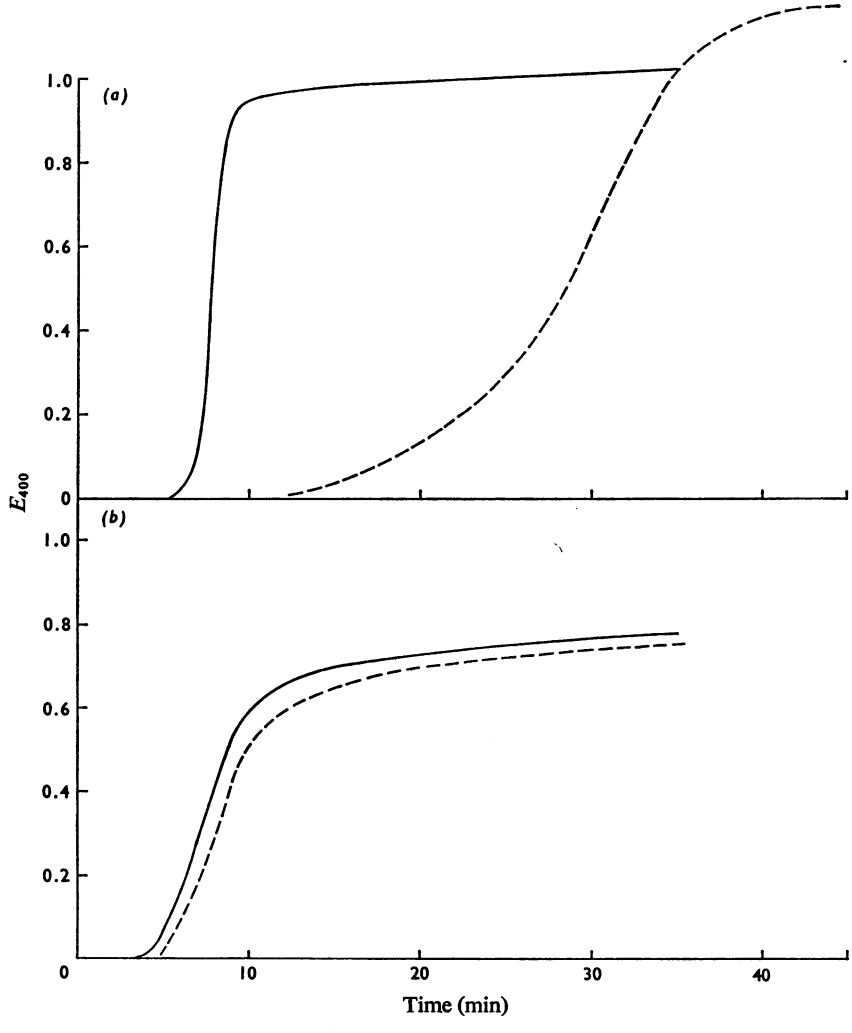

Fig. 2. *Effect of pepsin treatment on the rate of fibril formation*

Rates of fibril formation were followed by continuous monitoring at 400nm. All collagen solutions were at pH 7.2 and a concentration of 0.1%. The temperature was 38°C. In (*a*) collagens A and Ap are compared, in (*b*) collagens P and Pp. For details of the preparation of the collagens see the Experimental section. ——, Collagens A and P; – – – –, collagens Ap and Pp.

which solutions of collagen can form rigid gels containing native-type fibrils. Subsequently, other workers (e.g. Rubin *et al.*, 1963; Connel & Wood, 1964) have investigated the results of limited proteolysis on the rate of fibril formation. We therefore used a similar turbidimetric technique to test whether further pepsin treatment of collagens A and P resulted in a decrease of fibril-forming capacity. The results (Fig. 2) show that, whereas collagen A was markedly inhibited, pepsin treatment of collagen P was virtually without effect. The finding with collagen A was much as expected, but the failure to produce significant retardation of fibrillogenesis with collagen P was not expected, and suggests that sufficient quantity of a 'nucleus-forming' collagen remained despite removal of a large percentage of the covalent cross-links by pepsin. Although there is no direct evidence, it is tempting to suggest that this is related to the pepsin-resistant collagen demonstrated previously.

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