BIOPHYSICS AND BIOCHEMISTRY

Structural Modification of Collagen during Nonenzymatic Glycosylation *In Vitro*

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 133, No. 5, pp. 514-518, May, 2002
Original article submitted December 5, 2001

We studied changes in collagen resistance to proteases and formation of new fluorescent compounds during *in vitro* nonenzymatic glycosylation. The inhibitory effects of guanidine chloride and catalase were different: guanidine chloride inhibited cross-linking in collagen molecules, while catalase inhibited the formation of chromophore-containing compounds.

Key Words: *collagen; glycosylation; guanidine chloride; catalase*

Nonenzymatic reactions between carbohydrates and proteins (nonenzymatic glycosylation, NEG, or Maillard reaction) result in the formation of cross-links within and between protein molecules and modulate protein structure and functions [4,5,8].

End-products of glycosylation differ by chemical structure and are characterized by specific spectrofluorometric features [2,6,9].

NEG leads to protein aging and complicates the course of many diseases (diabetes mellitus, atherosclerosis, cataract, Alzheimer's disease, *etc*.) [10,13]. Pathological changes of the connective tissue (decreased skin elasticity, increased rigidity of vascular wall, *etc.*) determined by modifications of collagen structure accompany aging and diabetes mellitus [3,5,6,10,13]. NEG of collagen *in vivo* and *in vitro* attracts much recent attention, but specific changes in collagen structure are little studied.

We studied modification of collagen molecules in the course of NEG *in vitro* by evaluating protein sensitivity to proteases and spectrofluorometric characteristics of hydrolysates.

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MATERIALS AND METHODS

Commercial lyophilized acid-soluble collagen fraction (Serva) was used. Collagen was incubated for 1, 2, 5, and 12 months at 37°C under aerobic conditions in 1.5 M glucose in 0.2 M phosphate buffer (pH 7.4), for 12 months in phosphate buffer without glucose (control), and for 1 month with glucose and 0.3 M guanidine chloride (GC) or catalase (3000 U/ml) [4,5].

Collagen resistance to proteolytic enzymes was evaluated by hydroxyproline release at a 1:2 enzyme:substrate ratio (37°C, 18 h) [12]. Products of collagen proteolysis were analyzed spectrofluorometrically on a Shimadzu-RF 540 spectrofluorometer. Fluorescence intensity was measured at collagen concentration below 0.1 mg/ml and standardized to 0.1 mg/ml (after distraction of enzyme fluorescence).

The data were statistically processed using Student's *t* test for small samples [1].

RESULTS

Modification of collagen structure during *in vitro* NEG (cross-linking) was evaluated by protein resistance to proteolytic enzymes (Fig. 1). NEG considerably changes collagen resistance to pepsin and collagenase (re-

0007-4888/02/1335-0444$27.00

sistance to pepsin decreased most rapidly). Resistance to pronase little differed from the control.

These differences can be explained by specific effects of the studied proteases on collagen molecule. Collagenase attacks helical structures of collagen molecule [11]. Pepsin hydrolyzes terminal fragments of collagen molecule and does not affect its helical structures (this enzyme is active only at acid pH, *i. e.* under conditions of collagen denaturation [9]). Pronase also hydrolyzes terminal fragments of the molecule, but this enzyme is characterized by wider substrate specificity compared to pepsin [7], that is a greater variety of target points on the collagen molecule (even with NEG-produced cross-links).

NEG induces the formation of various fluorescent products [2,6,10] (imidasoles, pyrrols, furanes, pyrimidines, pyrasines, and other compounds with known spectral characteristics [4,6,8,10]).

Structural changes in collagen during *in vitro* NEG were evaluated by spectrofluorometric characteristics of its solutions after proteolysis with different enzymes. Fluorescence spectra of collagen hydrolysates were measured at excitation wavelengths of 335, 350, and 370 nm (Table 1).

Compounds fluorescing at 400-450 nm formed and accumulated during *in vitro* NEG throughout the experiment. It should be noted that hydrolysates of glycosylated collagen produced by different enzymes differed by fluorescence intensity. During the first month of NEG the greatest increment in chromophore-containing compounds was observed after collagenase and pronase proteolysis, the most pronounced difference was observed at excitation/emission wavelength of 335/400 nm corresponding to pentosidine fluorescence [2,10]. After 5- and 12-month NEG the maximum fluorescence was characteristic of collagen solutions treated with pepsin. It can be hypothesized that during the first month of protein-glucose reaction structural changes involved primarily helical structures, while at later terms chromophore-containing compounds more intensely formed in terminal parts of collagen molecules.

Hence, the resistance of glycosylated collagen to pepsin and collagenase characterizing intra- and intermolecular cross-linking increased during the first months of *in vitro* NEG, whereas fluorescence intensity (formation of chromophore-containing compounds) increased after long-term incubation with glucose.

GC inhibits the formation of end-products of protein glycosylation without affecting the formation of early products (aldimines, enol- and ketoamines) [4]. The effect of catalase is associated with inhibition of protein glycooxidation and glucose autooxidation requiring hydrogen peroxide [5]. In the presence of GC and catalase *in vitro* NEG of collagen was associated with less pronounced protein modification recorded by its resistance to proteases (Fig. 2). This was most demonstrative for pepsin, though similar results were observed with collagenase. GC produced a more prono-

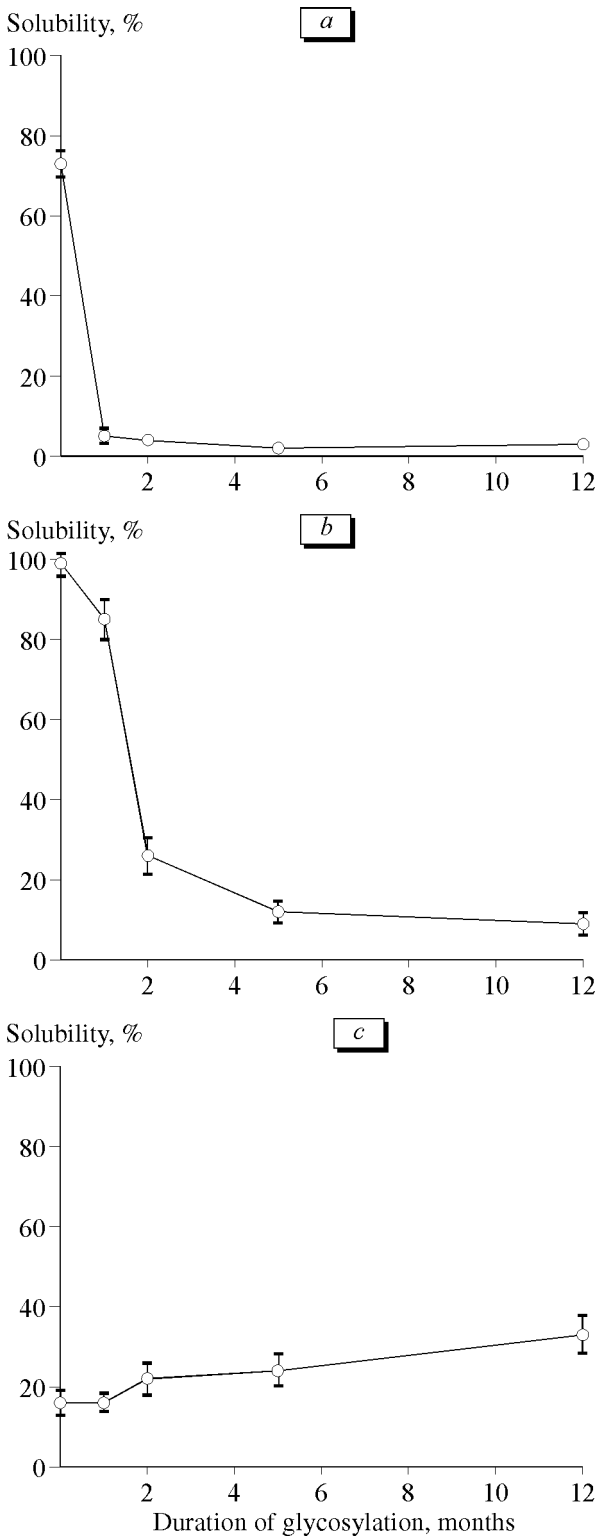

Fig. 1. Relationship between collagen solubility under the effects of pepsin (*a*), collagenase (*b*), and pronase (*c*) and duration of its glycosylation.

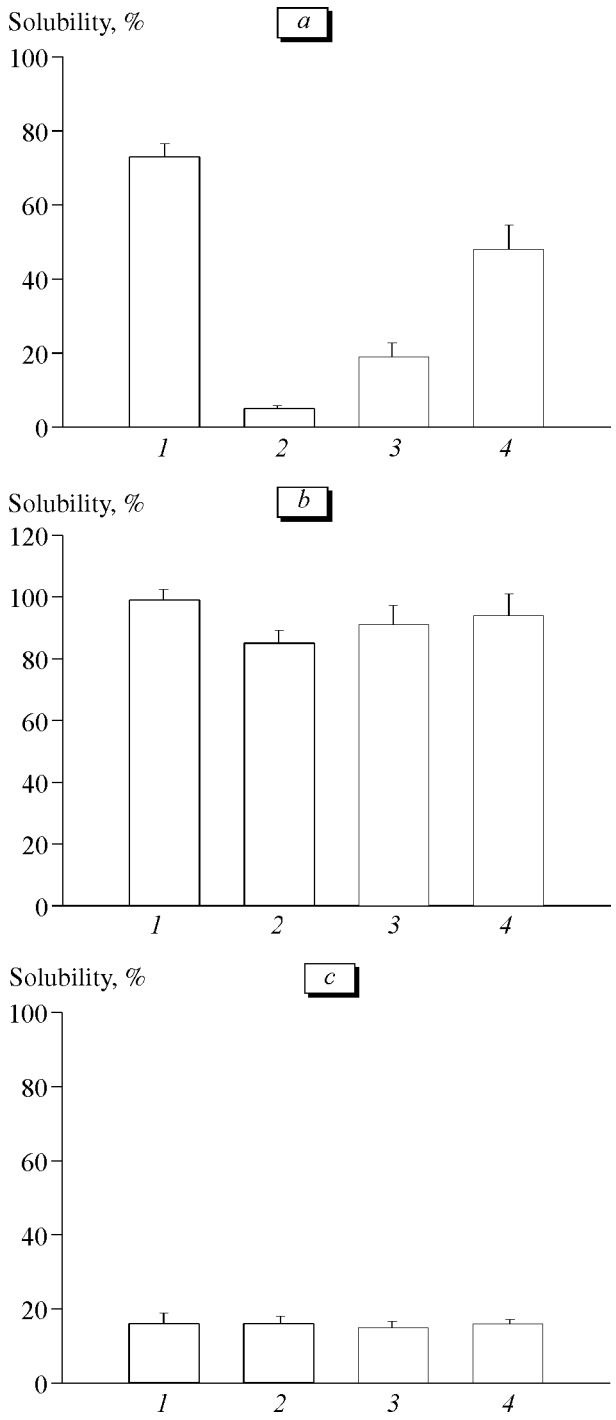

Fig. 2. Collagen solubility under the effects of pepsin (*a*), collagenase (*b*), and pronase (*c*) after 1-month incubation in phosphate buffer without glucose (*1*), with glucose (*2*), with glucose and catalase (*3*), with glucose and guanidine chloride (*4*).

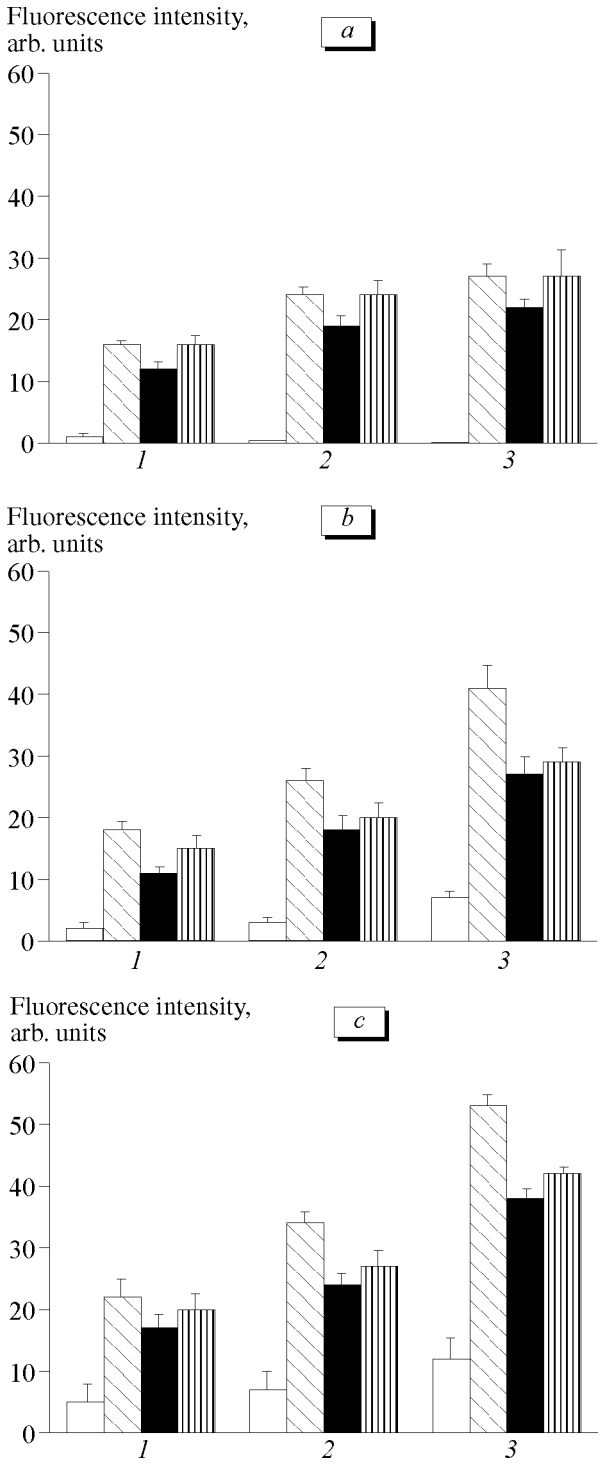

Fig. 3. Intensity of fluorescence of products of collagen proteolysis with pepsin (*a*), collagenase (*b*), and pronase (*c*) after 1-month incubation of collagen in phosphate buffer without glucose (light bars), with glucose (oblique cross-hatching), with glucose and catalase (dark bars), and glucose and guanidine chloride (vertical cross-hatching). Stimulation/emission wavelength: 370/440 (*1*), 350/420 (*2*), and 335/400 (*3*).

unced effect than catalase, which can be explained by the formation of different compounds. Solubility of modified collagen under the effect of pronase did not increase.

The study of the fluorescence intensity of collagen proteolysis products showed an inhibitory effect of GC and catalase on the formation of additional chromophore-containing compounds (Fig. 3). The most pronounced effects of inhibitors (decrease of fluorescence intensity) were observed for glycosylated

TABLE 1. Fluorescence Intensity (Arb. Units) of Collagen Proteolysis Products before (Control) and after Its Incubation with Glucose (*M*±*m*)

Enzymes; experimental conditions		Excitation/emission wavelength, nm		
		370/440	350/420	335/400
Collagenase				
control		2.0±0.4	3.0±0.3	7.0±0.5
glycosylation, months	1	18±1	26±2	41±4
	2	23±3	36±3	70±2
	5	47±4	78±3	116±15
	12	47±7	68±9	126±12
Pronase				
control		5.0±0.7	7.0±1.1	12.0±3.2
glycosylation, months	1	22±3	34±2	53±2
	2	23±2	43±2	86±2
	5	45±5	72±5	133±12
	12	47±3	72±2	141±7
Pepsin				
control		1.0±0.4	1.0±0.2	1.0±0.2
glycosylation, months	1	16±1	24±1	27±2
	2	33±6	59±6	95±1
	5	82±11	139±21	203±27
	12	87±5	134±5	181±4

collagen proteolysis products exposed to collagenase and pronase. Analysis of collagen hydrolysate fluorescence under the effect of pepsin showed no effect of GC, while the effect of catalase was less pronounced.

Comparative analysis of the inhibitory effects of GC and catalase in the course of collagen NEG showed a greater inhibitory effect of catalase on the formation of fluorescent compounds. Presumably, catalase decelerates protein glycooxidation and glucose autooxidation by decreasing the level of hydrogen peroxide.

Hence, our findings indicate that *in vitro* NEG is a multifactorial process, which is seen from the difference in the inhibitory effects of GC and catalase. GC more intensely inhibited the formation of additional cross-links, while catalase more actively suppressed generation of new chromophore-containing compounds in glycosylated collagen.

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