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N-glycosylation of proteins

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Abstract

Maillard reaction includes interaction of reducing sugar with any compound containing free amino group, which is resulted in formation of colored high-molecular compounds [1, 2]. Despite intensive research, a complete mechanism of Maillard reaction is still undefined. Primary product of reaction between sugars and amino acids, N-glycoside right after its formation experiences two types of transformations: 1. hydrolyzation into initial products and 2. rearrangement according to scheme offered by Amadori, which product is 1-amino-1-deoxy-2 ketose [3].

We have studied proteins N-glycosylation mechanism during Maillard reaction. Proteins mainly participate in Maillard reaction in the form of their free amino groups, to which ε-amino groups of lysin and α-amino groups of terminal amino acids belong.

Keywords: Maillard reaction; N-glycoside; Amino groups; Proteins.

1. Introduction

Maillard reaction includes interaction of reducing sugar with any compound containing free amino group, which is resulted in formation of colored high-molecular compounds [1, 2].

The mechanism of Maillard reaction was studied first by Hodge, who noted a huge complexity of interaction between sugars and amins and diversity of transformations participating in this process [3].

Reaction rate and nature of formed compounds are mainly determined by reaction conditions including chemical properties of carbohydrate and amine participating in reaction, reaction medium characteristics (pH and water content, presence of oxygen and metal ions), temperature and process duration, presence of reaction inhibitors (e.g. reducers) etc. [4].

Despite intensive research, a complete mechanism of Maillard reaction is still undefined. Primary product of reaction between sugars and amino acids, N-glycoside right after its formation experiences two types of transformations: 1. hydrolyzation into initial products and 2. rearrangement according to scheme offered by Amadori, which product is 1-amino-1-deoxy-2 ketose [3].

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We have studied proteins N-glycosylation mechanism during Maillard reaction. Proteins mainly participate in Maillard reaction in the form of their free amino groups, to which ε-amino groups of lysin and α-amino groups of terminal amino acids belong. In the process of food production industrial processing and storage Maillard reaction is a main reason of structural and chemical change of proteins [5]. Nutrient and functional properties of dietary proteins depend heavily on this reaction. As a result of Maillard reaction, intermolecular covalent bonds may form between protein molecules, i.e. cross-linking of protein molecules may occur which leads to loss of nutritional value of the latter, in addition, is causes food quality degradation and safety level decrease [6].

Under Maillard reaction conditions, use of labeled compounds during investigation of interactions between proteins and glucose provides significant advantage, since the process can be controlled not only according to free number of reacting components, but by means of labeled components entering into reaction.

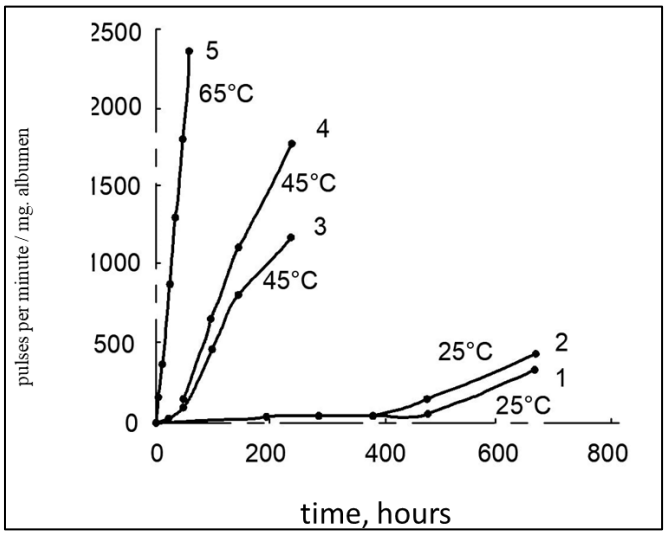
2. Experimental part

Some parameters of Maillard reaction between protein and aldoses have been studied by us on the example of bovine serum albumen glycosylation, when a glucose (1-6 ^{14}C -D-glucose with a specific radioactivity 909,1 Becquerel/ml. mole) labeled with radioactive carbon has been taken as a glycating agent. Bovine serum albumen is accessible in the form of pure homogenic protein and is widely used in biochemical experiments. Our interest to the mentioned protein has been stipulated by the fact that it contains free amino group in especially large amounts. In particular, an albumen molecule (M = 67000) contains 57 lysine- and 1 terminal free amino group.

With the purpose of N-glycosylation, 1-6 ^{14}C -D-glucose has been added to albumen. We have taken 9,36 mg or 46,8 mg of glucose per 60 mg of albumen, reaction has been carried out in 30 ml of 0,06M phosphate buffer (pH = 5,0; 7,0; 8,0), in darkness, at T = 25°C; 45°C; 65°C. In specified intervals samples in the amount of 5 ml have been taken from reaction mixture, which have been dialyzed in special dialysis bags SERVAPOR® (which detains proteins with ≥12000 molecular mass). Optimum duration of dialysis has been established via everyday measurement of dialysable samples radioactivity. Formation of N-glycosylated products has been controlled via determination of radioactivity of 1 ml of dialyzed solution. In parallel, such tests have been conducted based on albumen and unlabeled D-glucose in order to determine color formation intensity and amount of free amino acids in glycosylated albumen. Amount of 1-6 ^{14}C -D-glucose in N-glycosylated albumen hydrolysate has been determined by autoradiography [7].

We have determined amino acetylated albumen necessary for our experiments according to the known method [8]. 1 mole of obtained amino acetylated albumen contained 55,6 moles of acetyl group. We have determined primary amino groups [9] and acetyl groups in albumen and amino acetylated albumens [10].

Effect of temperature and albumen-glucose molar ratio on N-glycosylation reaction running at pH = 7,0 is given in Figure 1.



1,3,5 – equivalent proportion of albumen-glucose = 1:1; 2,4 - equivalent proportion of albumen-glucose = 1:5

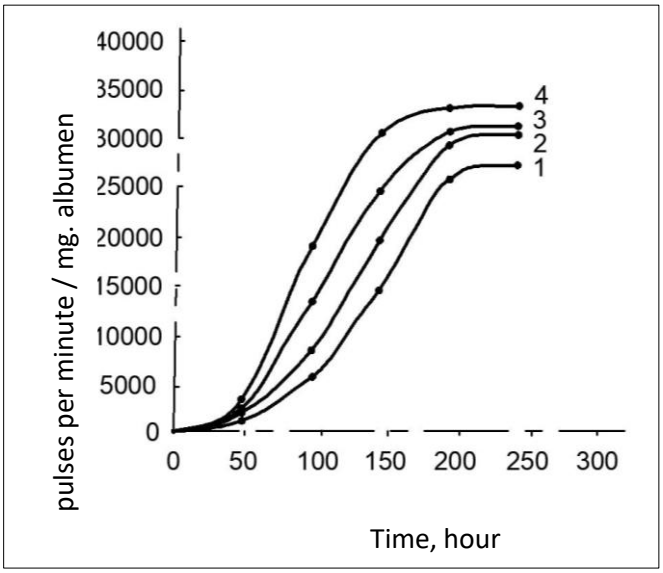
Figure 1 Effect of temperature and reacting components' equivalent proportion on irreversible inclusion of 1-6 ^{14}C glucose carbon into albumen (0,06 M phosphate buffer, pH = 7,00)

Under relatively mild reaction conditions (T ≥ 50°C and moderately abundant quantity of glucose), albumen may participate in Maillard reaction only at the expense of lysine ε-amino groups and terminal amino groups available in it. Molecular mass of albumen used by us consists 67000, while a number of free amino groups equals to 58 according to our analyses (amine nitrogen 1.2%).

Based on this fact, 1 albumen equivalent comprises 1155 in this reaction. Fig. 1 shows that under conditions of T = 25°C and equivalent proportion of albumen-glucose 1:1, during first 500 hours of incubation there is virtually no inclusion of glucose labeled carbon into albumen composition (Fig. 1, curve 1); in case of glucose concentration increase (equivalent proportion of albumen-glucose 1:5), a degree of irreversible glucose inclusion substantially rises (Fig. 1, curve 2), though inclusion degree in both cases is very low.

Reaction mixture temperature rise up to 45°C substantially boosts the irreversible inclusion process (Fig. 1, curves 3, 4), while at 65°C temperature the process intensity sharply increases even in case of reagents proportion 1:1 (Fig. 1, curve 5). Thus, temperature rise has much stronger effect on Maillard reaction rate then molar ratio of reacting components.

The intensity of Maillard reaction between albumen and glucose increases with rise of reaction medium pH, as is seen in Fig. 2. The degree of labeled carbon inclusion in albumen increases with gain in glucose concentration (Fig. 2, curve 4). In our experiment we have used low glucose concentration, that is why relatively intense formation of melanoidin coloring has been registered at 65° temperature only, in neutral and basic medium. Interesting regularity has been recorded in these experiments: radioactivity measurements have showed that after 192-hour exposition within 192-240-hour interval, degree of ^{14}C inclusion into albumen has been never increased, but despite this fact, the intensity of melanoidin coloring of reaction mixture has substantially increased.



1 – equivalent proportion of albumen-glucose = 1:1, pH 5,0; 2 – equivalent proportion of albumen-glucose = 1:1, pH 7,0; 3 – equivalent proportion of albumen-glucose = 1:1, pH 8,0; 4 – equivalent proportion of albumen-glucose = 1:5, pH 8,0.

Figure 2 Effect of pH and reacting components' equivalent proportion on irreversible inclusion of 1-6 ^{14}C glucose carbon into albumen (0,06 M phosphate buffer, 65°C)

Maillard reaction between glucose and albumen is characterized by an initial induction period, which is getting smaller with rise of reaction temperature (Fig. 1 and 2). Induction period is followed by a lengthy period, during which the degree of ^{14}C inclusion into albumen almost linearly increases with time (Fig. 1). Previously conducted spectrophotometric measurements also have pointed at the existence of such induction period during glucose-albumen interaction. This induction period corresponds to N-glycoside formation phase. [11] N-glycosides easily hydrolyze even in the neutral medium, so the initial phase of glucose-albumen interaction is reversible: N-glycosidic bond is hydrolyzed during dialysis and a glucose molecule is detached from albumen molecule. In parallel with hydrolysis process, N-glycoside experiences Amadori rearrangement, as a result of which amino ketose is formed first, and afterwards – many other compounds "final products of glycation or else glycosylation", and this reaction period corresponds to irreversible insertion of glucose molecule or its fragments into albumen. [12]

As is seen from the Fig. 2, under given conditions of N-glycosylation reaction, the moment of saturation is reached after definite period of time and, albumen doesn't link up any more glucose. Table 1 data show that this saturation moment comes despite the fact that there is still a sufficient amount of free amino groups left in albumen necessary for glucose binding.

3. Discussion of the obtained results

In our experiments, a minimal degree of N-glycosylation (0,6% of glycosylated primary amino groups in albumen) has been registered under conditions of equivalent proportion of albumen-glucose = 1:1, at 25°C temperature and pH – 7,0, when reaction period was 672 hours. A maximal degree of N-glycosylation has been registered at equivalent proportion of albumen-glucose = 1:5, 65°C temperature, pH – 8,0 in case of 240-hour duration (Table 1). It should be noted that resulting from 40-day incubation (37°C, phosphate buffer, pH 7.4) of bovine serum albumen and glucose mixture a protein was obtained, in which the glycosylation degree of lysine ε-amino groups comprised 75,7% of total amount of free amino groups [13].

Table 1 Amount of tie 1-6 ^{14}C-D glucose and N-glycosylated primary amino groups in albumen

Glycosylated albumen preparation conditions				**Albumen**	
Albumen-glucose, equiv. proportion	pH	T, ^{0}C	time, hours	Attached glucose, mg/g	Glycosylated primary amino groups % (according to tie 1-6 ^{14}C)
1:1	7.0	25	672	1.0	0.6
1:5	7.0	25	672	1.3	0.8
1:1	7.0	45	480	7.3	4.7
1:5	7.0	45	480	11.3	7.2
1:1	7.0	65	240	94.0	60.3
1:1	7.0	65	240	103.6	66.4
1:1	8.0	65	240	106.6	68.3
1:5	8.0	65	240	113.3	72.6

Table 2 Glucose formation resulting from hydrolysis N-glycosylated albumen (N-glycosylated albumen is received under following conditions: equivalent proportion albumen-glucose = 1:5, 65°C, pH - 8.0, 240 hours)

Hydrolyzing agent	**Temperature, °C**	**Time, hours**	**Glucose, %**
0.1 N HCI	25	24	0
0.1 N HCI	25	48	trace
0.1 N HCI	100	9	0.5
1 N HCI	100	12	2.8
1N HCI	100	24	4.3
6 N HCI	100	24	0.7

As a result of free amino groups' blocking a protein loses the glucose binding ability. We have implemented acetylation of free amino groups available in albumen and have studied the ability of acetylated protein compound with acetylated protein. Analysis has showed that 1 mole of acetylated albumen contained 55.6 moles of acetyl groups. Thus, almost 95% of free amino acids have been blocked. In order to eliminate hydrolytic detachment of acetyl groups, the glycosylation process of acetylated albumen has been studied by us in neutral medium, at 45°C and 65°C temperatures. It turned out that irreversible inclusion of glucose molecules or its fragments into albumen doesn't occur under these conditions.

Study of products of acidic hydrolysis of albumen N-glycosylation products has showed that a glycolyzed albumen contains a part of glucose in hydrolyzed form, though as can be seen, the basic part of labeled glucose is irreversibly included into albumen in the form of different products of included glucose's transformation (Table 2).

Regarding the data represented in Table 2, one must take into account that a glucose intensely transforms when heated in strongly acid medium. Probably, this fact explains its small amount in hydrolysate obtained with the use of 6 N HCl.

4. Conclusions

It is established, that in the protein glycosylation process (bovine serum albumen ([1-6 ^{14}C] – D-glucose)) the reaction intensity increased with growth of reaction medium pH. Degree of labeled carbon inclusion into albumen increases with growth of glucose concentration. Reaction is characterized by initial induction period, which reduces with reaction temperature rise. Induction period is followed by a lengthy period, during which a degree of ^{14}C inclusion into albumen almost linearly increases with time. After definite period of time the moment of saturation is reached and despite availability in albumen of sufficient amount of free amino groups necessary for glucose binding, albumen no more links up any more glucose.

Resulting from free amino groups blocking (acetylated albumen) protein loses the glucose attachment ability. N-glycosylated albumen contains part of glucose in hydrolyzed form, but the main part of labeled glucose is irreversibly included into albumen, in the form of different products of glucose transformation.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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