

Industry

GLYCATION OF PROTEINS IN *ESCHERICHIA COLI*: EFFECT OF NUTRIENT BROTH INGREDIENTS ON GLYCATION

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ABSTRACT

*Glycation is a condensation reaction between reducing sugars and primary amino groups in proteins. It starts with formation of Schiff bases and Amadori products (called early glycation products), which are later converted into complex heterocyclic compounds designated as advanced glycation end (AGE) products. Until recently glycation was studied mainly in higher eukaryotes and only our recent studies [6] showed that glycation took place also bacteria. The aim of this study is to investigate the effect of growth media and nutrient broth ingredients on glycation of proteins in *Escherichia coli*. The obtained results indicate that glycation is more extensive in rich media and depends on the type and source of nutrient broth ingredients (yeast extract and protein hydrolysate). Sometimes different batches of one ingredient produced by the same manufacturer might have different effect on glycation. Since the increased glycation decreases stability and biological activity of recombinant proteins, we highly recommend all nutrient broth ingredients to be tested for their effect on glycation before use for manufacturing of such proteins.*

Introduction

Glycation or non-enzymatic glycosylation is a condensation reaction between reducing sugars and primary amino groups in proteins. It starts with formation of Schiff bases, which are consecutively converted into ketosamines (Amadori products) and complex heterocyclic compounds designated collectively as advanced glycation end products (AGEs) (1-3). Besides in food chemistry, AGEs are extensively studied in clinical biochemistry in relation with the diabetes and aging. In principle, until recently, main objects for studying glycation were higher eukaryotes and man. There are only few reports on glycation in lower eukaryotes (4, 5) and, until recently, no reports on glycation in prokaryotes. Applying specific mon-

oclonal antibodies against AGEs, fluorescent spectroscopy, mass-spectrometry and other adequate methods for protein research, we have reported for the first time that glycation takes place also in *Escherichia coli* (6). Unlike enzymatic glycosylation, which is designed by nature to make proteins functional and stable, glycation is destructive for biopolymers. In a recent study we have shown that recombinant human interferon- γ (rhIFN- γ) isolated from *Escherichia coli* is partly glycated and that the glycated fraction undergoes severe molecular changes including non-enzymatic degradation, covalent dimerization and loss of biological activity (6, 7). That is why it is important for recombinant DNA biotechnology to know the factors affecting glycation in bacteria.

This study aims to investigate the effect of nutrient broth ingredients on glycation of proteins in *Escherichia coli*.

Materials and Methods

Nutrient broth ingredients

Nutrient broth ingredients (protein hydrolysates and yeast extracts) were purchased or donated from different sources (some of the companies do not exist anymore) such as Gibco (USA), Difco (USA), Scharlau (Germany), BBL (UK), Fluka (Switzerland), Merck (Germany), Microbiologie (France), etc.

Growth media

Luria-Bertani (LB) medium contains 1% protein hydrolysate, 0.5% yeast extract and 0.5% NaCl adjusted to pH 7.4.

The minimal (M9) medium contains: 40 µg/ml amino acid mix, 0.5 µg/ml thiamin, 1 mM MgSO₄ and 0.2% glucose adjusted to pH 7.4.

Isolation of total bacterial protein

Nutrient broth was inoculated with an overnight bacterial culture of *E. coli* AB1157 and cultivated at 37°C to middle exponential phase. The cells were harvested by centrifugation and lysed by ultrasonication in 10 mM Tris-HCl, pH 7.5, 0.15 NaCl and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The lysates were cleared by centrifugation and supplemented with 10 mM MgCl₂, DNase I and RNase A (50 µg/ml each) and lysozyme (20 µg/ml). Following incubation at 37°C for 1 h, the low molecular weight material was removed by gel filtration on Sephadex G25 column and the total protein was used for analysis.

Determination of early (Amadori) glycation products

Early glycation products (ketosamines or Amadori products) in total bacterial protein were determined by the method of Johnson *et al.*, (8) modified as follows: 0.1 ml protein samples (5 mg/ml) were mixed with 1

ml 0.1 M carbonate-bicarbonate buffer containing 204 mg/l nitroblue tetrazolium (NBT) and incubated at 37°C for 45 min. The content of Amadori products (nM/ml) was determined by multiplying the absorbance at 530 nm by the coefficient 12.64.

Determination of late glycation products (AGEs)

Fluorescent AGEs were determined by fluorescence measurement on a Shimadzu RF-5000 spectrophotometer at $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 440$ nm and non-fluorescent AGEs were quantified by ELISA using a monoclonal antibody against N-carboxymethyllysine (CML).

Results and Discussion

As already mentioned, the non-enzymatic glycosylation in bacteria has been discovered three years ago [6] and due to its recency the bacterial glycation agents are not identified yet. Although there is a reason to assume that these substances are related with the normal bacterial metabolism and therefore are endogenous products, we can not ignore entirely their hypothetical exogenous origin. To shed light on this matter, we have designed to investigate in this study the effect of nutrient broth composition on the glycation in *E. coli*. Taking into consideration that the non-enzymatic glycosylation is a multistage process, we designed to analyse total protein isolated from bacteria grown in different broths for content of both early and late glycation products. Early glycation products (ketosamines or Amadori products) were determined by the NBT method and presented in nM/ml fructosamine. Since the late products (AGEs) are divided into two types - fluorescent and non-fluorescent, total bacterial protein was analysed for both. Fluorescent AGEs were determined by their specific fluorescence at 440 nm upon excitation at 360 nm and the non-fluorescent compounds were quantified by ELISA us-

TABLE 1

Early and advanced glycation end products in total bacterial protein isolated from *E. coli* AB1157 cultivated in rich (LB) and minimal (M9) nutrient broths

Nutrient broth	Fructosamine (nM/ml)	CML*(mg/ml)	FI(arbitrary units)
LB	1.73 ± 0.11	1.75 ± 0.19	32.83 ± 1.50
M9	1.17 ± 0.08	0.41 ± 0.05	23.48 ± 1.08

*Content of CML in total bacterial protein responds to content of CML in *in vitro* glycosylated BSA (mg/ml).

ing a monoclonal antibody specific to the most typical non-fluorescent glycation end product - N-carboxymethyl lysine (CML).

Effect of the medium type (rich or minimal) on glycation

Both rich and minimal media are widely used in microbiology and biotechnology. To study the effect of medium type on glycation, *E. coli* AB1157 cells were cultivated in LB (rich) and M9 (minimal) media and their protein was analysed for both early and late glycation products. The results presented in Table 1 show that the content of both substances was higher in the cells cultivated in rich medium. For the Amadori products and fluorescent AGEs the difference was about 1.5 times in favor of the cells cultivated in LB medium, whereas for the non-fluorescent AGEs (CML) this difference was as high as 4 times. Since the M9 medium contains reducing sugar (glucose) and the LB medium is sugar free, this result indicates that the glycating agents of *E. coli* are endogenous products and their biosynthesis is favored in rich nutrient broths.

Effect of nutrient broth ingredients on glycation

The two main ingredients of the rich LB growth medium are yeast extract and protein hydrolysate. They both were varied in our experiments to study the effect of some variables such as source, age, etc. on glycation of proteins in *E. coli*. To this end one of

the ingredients (either yeast extract or protein hydrolysate) was kept constant and the other one was varied. Ingredients were purchased from different companies (see Materials and Methods). Sometimes different lots of one and the same company were also compared. The effect of the nutrient broth ingredients on glycation was evaluated on the basis of content of early and advanced glycation end products in total bacterial protein obtained from *E. coli* AB1157.

To examine the effect of yeast extract on glycation, eight different batches of yeast extract were combined in LB medium with Difco Bacto-Tryptone and inoculated with *E. coli* AB1157. The results from the analysis for content of glycation products in the total bacterial protein are presented in Fig. 1. As seen from Fig. 1A, the variations in content of early glycation (Amadori) products was negligible when different yeast extracts were used. Unlike the Amadori products, the variation in the content of AGEs was remarkable and was better expressed in the case of the non-fluorescent AGEs (CML). Fig. 1B shows that the level of CML corresponding to four of the yeast extracts (Gibco 13E5133, BBL, Difco 86898 and Microbiologie) was about twice higher in comparison with the other samples. As for the fluorescent AGEs (see Fig. 1C), the variation in their content was also negligible except for one sample (BBL), where the fluorescent intensity was

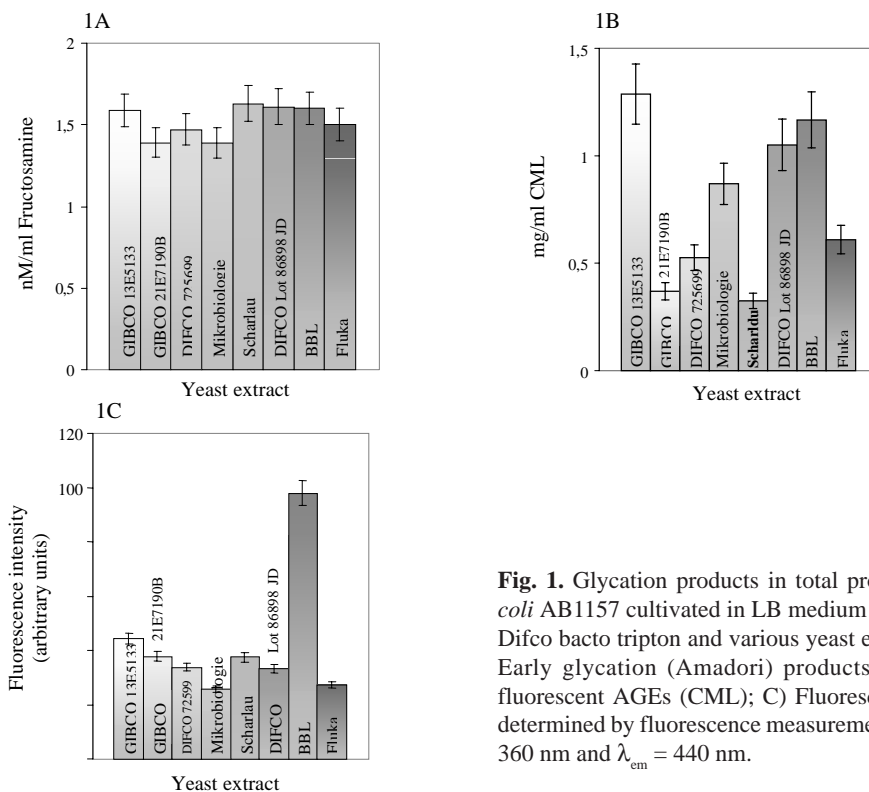


Fig. 1. Glycation products in total protein of *E. coli* AB1157 cultivated in LB medium containing Difco bacto tripton and various yeast extracts. A) Early glycation (Amadori) products; B) Non-fluorescent AGEs (CML); C) Fluorescent AGEs determined by fluorescence measurements at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 440$ nm.

2.0-2.5 times higher compared to that of the other seven samples.

To study the effect of the type and source of protein hydrolysate on glycation, eight different protein hydrolysates (both tryptones and peptones) were combined in LB medium with Scharlau yeast extract and inoculated with *E. coli* AB1157. The results from the analysis of the total bacterial protein are presented in **Fig. 2**. As in the case of yeast extracts (see above), the variations in type, source and age of protein hydrolysates did not interfere considerably with the formation of early glycation (Amadori) products (**Fig. 2A**). The effect of these variables was better expressed in the case of AGEs (**Fig. 2B and 2C**). As seen in Fig. 2B, some batches of tryptone (Scharlau and Difco) gave

more than three times higher content of CML than the others. On the other hand, some batches of peptone (BBL and Difco) yielded 1.5-2.0 times more fluorescent AGEs in comparison with the other hydrolysates.

The results presented in this study indicate that the glycation activity of *E. coli* is variable and depends on the type and composition of growth medium used. In principle, it is higher in rich media and depends on the type and source of nutrient broth ingredients. Our results show that different batches of one ingredient originating from one and the same manufacturer might have different properties. Bearing in mind that the increased glycation activity decreases stability and biological properties of recombinant protein obtained from bacteria, we highly

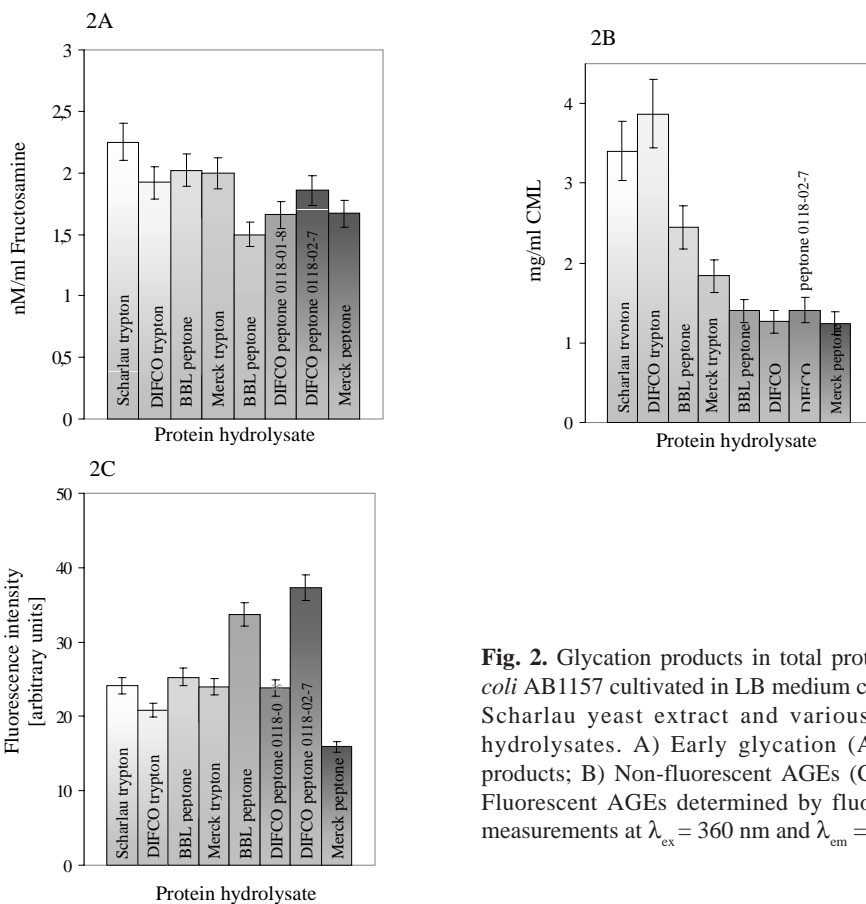


Fig. 2. Glycation products in total protein of *E. coli* AB1157 cultivated in LB medium containing Scharlau yeast extract and various protein hydrolysates. A) Early glycation (Amadori) products; B) Non-fluorescent AGEs (CML); C) Fluorescent AGEs determined by fluorescence measurements at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 440$ nm.

recommend all nutrient broth ingredients to be tested for their effect on glycation before use. This is particularly important when they are to be used in large-scale fermentation processes.

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