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Reactivity and oxidative potential of fructose and glucose in enkephalin-sugar model systems

Short Communication

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Summary. The reactions of Leu- and Met-enkephalin (Tyr-Gly-Gly-Phe-Leu/Met) with fructose resulted in the parallel formation of Heyns compounds (*N*-peptidyl-D-mannosamine and -D-glucosamine) and sugar-peptide generated imidazolidinone diastereomers. Glucose showed higher level of reactivity than fructose with respect to the extent of glycated product formation. The presence of fructose in the incubation mixtures makes Met residue more susceptible to oxidation than glucose.

Keywords: Enkephalin – Fructose – Glucose – Glycation – Heyns – Imidazolidinone – Maillard

Introduction

Fructose is a potent reducing sugar that participates in the formation of toxic advanced glycation products, which appear to play a role in aging processes as well as in the pathogenesis of the vascular, renal, and ocular complications of diabetes (Hinton and Ames, 2006; Schalkwijk et al., 2004). In addition, excessive fructose consumption may have a major role in the present epidemic of metabolic syndrome and obesity, due to its ability to raise uric acid (Nakagawa et al., 2006; Eavel, 2005; Gaby, 2005). According to previous studies (Levi and Werman, 2003; Suarez et al., 1989), incubation of proteins with fructose, at physiological temperature, resulted in faster accumulation of protein bound fluorescence and cross-linking than in analogues experiments involving glucose.

According to our knowledge, only two studies have been conducted on the formation and structural characterization of the peptide-derived glycation products from fructose (Heyns and Rolle, 1959; Linetsky et al., 2006). Given the significance of fructose-mediated modifications of proteins, we conducted this project (1) to examine the reaction products derived from fructose and enkephalins, endogenous peptides with potent biological activity (Sharp, 2006; Zagon and McLaughlin, 2006; Bodnar and Klein, 2005), (2) to determine whether fructose, as compared to glucose, is a much more potent initiator of the glycation reaction and (3) to study the effect of fructose/glucose concentration on the kinetics of sulfoxide formation from Met-enkephalin.

Materials and methods

*Synthesis of glycation products **3–6***

D-Fructose (270 mg, 1.50 mmol), peptide **1** or **2** (0.1 mmol) and *N*-ethylmorpholine (NEM) (192 µl, 1.50 mmol) were dissolved in dry MeOH (20 ml) and the reaction mixture was stirred for 24 h at 70 °C. Purification by RP HPLC using 40% MeOH/0.1% TFA as the eluent afforded pure glycated compounds: **3** (11 mg, 15%; $t_R = 14.21$ min) and **4** (Isomer 1:10 mg, 14%; $t_R = 20.13$ min; Isomer 2:16 mg, 22%; $t_R = 22.60$ min) from Leu-enkephalin (**1**) and **5** (7 mg, 10%; $t_R = 8.85$ min) and **6** (Isomer 1:16 mg, 21%; $t_R = 11.57$ min; Isomer 2:20 mg, 27%; $t_R = 12.62$ min) from Met-enkephalin (**2**).

Incubations in phosphate buffer and MeOH

For incubations in PBS, peptide **1** (0.01 M) and reducing sugars, D-fructose or D-glucose (0.01, 0.15 or 1.50 M) were prepared in 0.05 M phosphate buffer/0.1 M NaCl (pH 7.4) (PBS) containing NaN_3 (0.02%). The sterile solutions, obtained by passage through a 0.45 µm nylon filter, were incubated in the dark at 37 or 50 °C. Aliquots were withdrawn from the incubation mixtures at appropriate time intervals, immediately frozen and lyophylized. The relative concentrations of the respective glycation products in the incubation mixtures were determined by analytical RP HPLC at a flow rate of 0.5 mL/min. The mobile phase used for the analysis

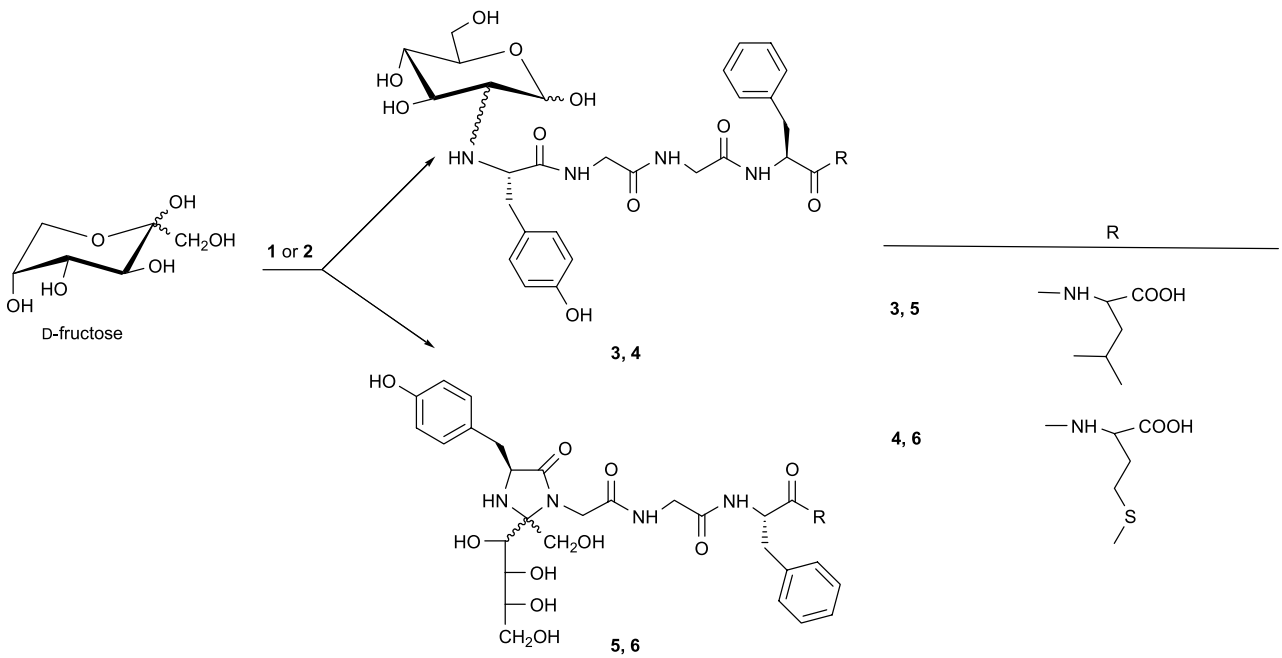
was: 43.5% MeOH/0.1% TFA. For reactions in MeOH, peptide **1** (0.001 M) and reducing sugars, D-glucose or D-fructose (0.015 M) were incubated in pure solvent or solvent containing *N*-ethylmorpholine (NEM) (0.050 M). The reaction mixtures were kept at 50 °C. The sampling procedure and analysis of products was performed as described above.

For measurement of the oxidative damage, Met-enkephalin (**2**) (0.01 M) and D-fructose or D-glucose (0.01, 0.15 or 1.50 M) were prepared in PBS containing NaN_3 (0.02%). The sterile solutions, obtained by passage through a 0.45 μm nylon filter, were incubated in the dark at 37 °C. The relative concentrations of the peptide **2**, Met-enkephalin sulfoxide and respective glycation products in the incubation mixtures were determined by analytical RP HPLC by using 40% MeOH/0.1% TFA as the mobile phase.

Results and discussion

The reaction of D-fructose (Fru) with the amino group of either Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu, **1**) or Met-enkephalin (Tyr-Gly-Gly-Phe-Met, **2**) in MeOH as the solvent led to the parallel formation of Heyns compounds, obtained as a mixture of *N*-(2-deoxy-D-glucopyranos-2-yl)- and *N*-(2-deoxy-D-mannopyranos-2-yl)-Tyr-Gly-Gly-Phe-Leu/Met (**3** or **4**), accompanied by imidazolidinone-ring-containing compounds **5** or **6** (Scheme 1). The imidazolidinone compounds **5** or **6** with D-*arabino* sugar tetritol structures attached to the ring were obtained as diastereoisomers having *R* or *S* configuration at the new *N*,*N*′-ketal center formed.

The reactivities of fructose and glucose with respect to their abilities to react with the primary amino group of Leu-enkephalin (**1**) were compared in phosphate buffered saline (PBS) (pH 7.4) or in MeOH. As shown in Table 1, in PBS the rate of glycation was very slow even after a 4-wk incubation (entry 1). However, the yield of glycation products increased with the sugar concentration in both model systems, glucose producing higher amounts of glycated products than fructose (entries 3–6). In the case of glucose, the reaction with pentapeptide **1** leads primarily to Amadori compound **7** [*N*-(1-deoxy-D-fructos-1-yl)-Tyr-Gly-Gly-Phe-Leu], whereas formation of the corresponding imidazolidinone **8** (Roščić and Horvat, 2006) was dependent on the glucose concentration. In the case of fructose, the glycation reaction resulted in higher amounts of imidazolidinone compound **5**. The ratio of **5**/**3** was dependent both on the amount of fructose and on temperature. In contrast to studies performed in buffered system, in the **1**-sugar-MeOH system, the reactivity of glucose was about 5-fold greater than that of fructose, Amadori derivative **7** being formed almost exclusively (entry 7). Addition of NEM increased the content of imidazolidinone compound **5** in the **1**/Fru system almost three times, while the amount of Heyns products **3** remained unchanged. Exposure of peptide **1** to glucose in MeOH-NEM medium resulted in almost equal amounts of glycation products **7** and **8** (entry 8). Although it has been reported that fructose is more effective in causing protein cross-linking and in generating protein-bound Maillard fluorescence than glucose, this study demonstrates that with respect of early glycation product formation, glucose



Scheme 1. Glycation products derived from Tyr-Gly-Gly-Phe-Leu (**1**) and Tyr-Gly-Gly-Phe-Met (**2**) in the presence of D-fructose

Table 1. Yields of the glycation products obtained by the reactions of D-fructose and D-glucose with Leu-enkephalin (**1**) under identical conditions, in phosphate buffered saline (pH 7.4) (PBS) or in MeOH

Entry	Sugar:**1** molar ratio	Solvent	Temp. (°C)	Incubation time (days)	Yields (%)[a]			
					Fructose		Glucose[b]	
					3	**5**	**7**	**8**
1	1:1	PBS	37	28	2	2	2	–
2	1:1	PBS	50	10	1	1	2	–
3	15:1	PBS	37	28	3	2	8	3
4	15:1	PBS	50	10	2	11	11	5
5	150:1	PBS	37	28	5	13	26	6
6	150:1	PBS	50	10	4	18	34	7
7	15:1	MeOH	50	7	6	7	61	1
8[c]	15:1	MeOH	50	7	6	24	26	22

[a] The concentration of glycated products obtained from **1** was measured by RP HPLC
[b] Data taken from Roščić and Horvat (2006)
[c] Yields of the glycation products in the presence of 50 equiv. of *N*-ethylmorpholine

is more effective in pentapeptide **1** *N*-alkylation than fructose.

To determine the effect of fructose and glucose concentration on the formation of the oxidized methionine residue, Tyr-Gly-Gly-Phe-Met (**2**) was incubated in PBS (pH 7.4) for 4 wk at 37 °C with various sugar concentrations under oxidative conditions. As shown in Fig. 1, Met-enkephalin sulfoxide, Tyr-Gly-Gly-Phe-Met(O), was formed readily, and in significant yield, in both sugar systems, but much higher yields of oxidation product were obtained after 1 wk in the **2**/Fru system. Based on RP HPLC analyses, and the fact that only traces of glycation products **5** and **6** were found in the reaction mixtures, after 1 wk of incubation, we concluded that the formation of Met-enkephalin sulfoxide could be ascribed to the sugar alone. As presented in Fig. 1, there was a strong correlation between the **2**-sulfoxide content and sugar concentration in incubation mixtures, including the trend to a plateau at the highest sugar concentration. The results indicate that oxidative damage to Met-enkephalin is most probably caused by a hydroperoxide intermediate formed from fructose or glucose *via* reaction of the respective enediolate anion with molecular oxygen in aqueous alkaline media (Arts et al., 1997).

In conclusion, this study demonstrates that reaction of fructose with the endogenous opioid peptides, Leu- and Met-enkephalin, results in two different types of products: *N*-(2-deoxy-D-aldopyranos-2-yl)-derivatives (Heyns compounds) and imidazolidinone ring-containing compounds. The obtained results indicate that, in buffered aqueous and

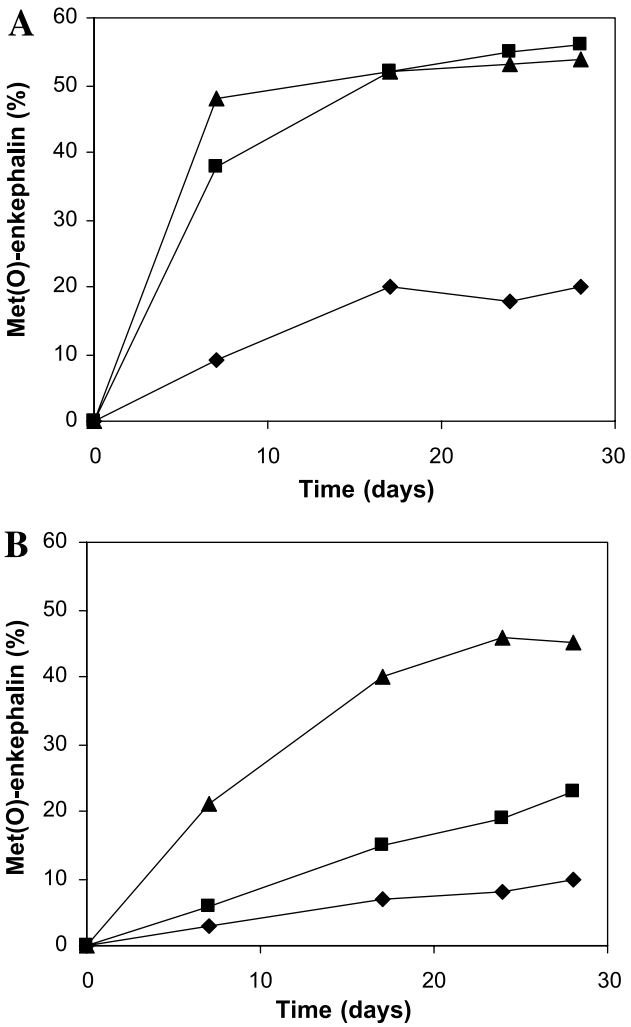


Fig. 1. Kinetics of Met-enkephalin sulfoxide formation in PBS, pH 7.4, at 37 °C, in the presence of 0.01 M (◆), 0.15 M (■) and 1.50 M (▲) D-fructose (**A**) and D-glucose (**B**)

methanolic solution, D-glucose is more reactive than D-fructose, with respect to the formation of *N*-glycated peptides. However, fructose has the higher oxidative potential to damage methionine residues in peptides.

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