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Effect of Monosodium Glutamate on the Digestibility of Different Nutrients Using Standardized Static In vitro Digestion Model

M.Yasser Alsedfy[1], Alaa Hassan Said[1], A.A. Ebnalwaled[1], Mona Moustafa[2]

[1]Electronics and Nano Devices Lab, Faculty of Science, South Valley University, Qena, 83523, Egypt
[2]Physics Department, Faculty of Science, Minia University, Egypt

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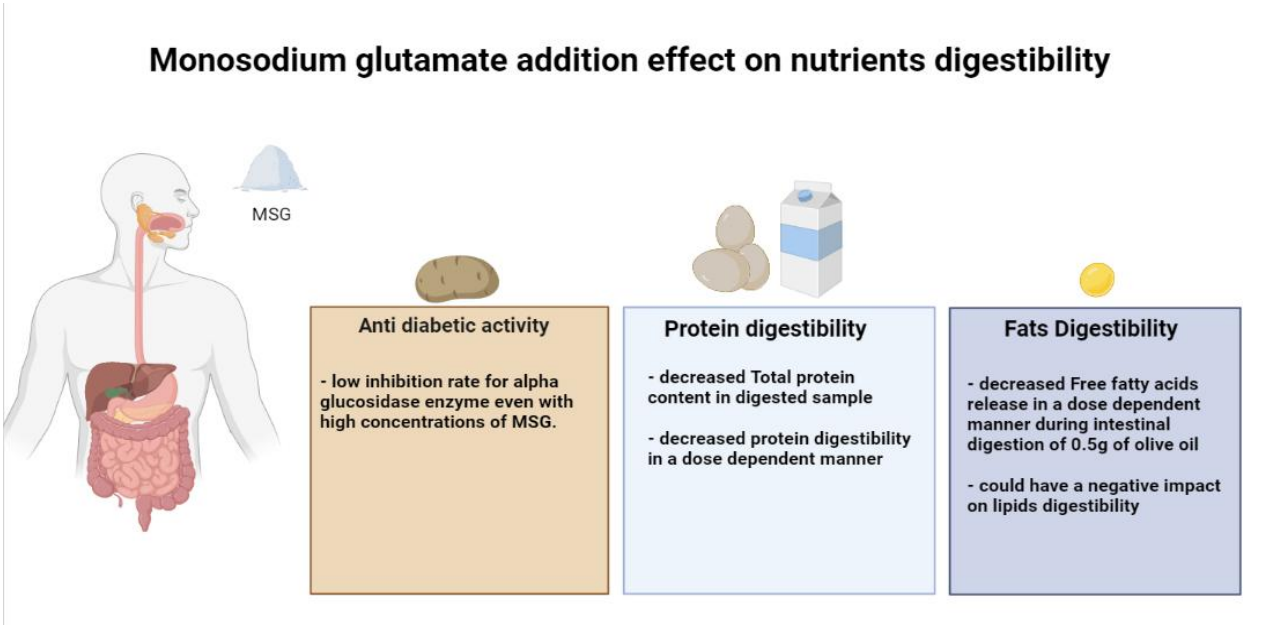
GRAPHICAL ABSTRACT



* Corresponding author
E-mail: muhammadyasseralhussainy@gmail.com (M. Yasser Alsedfy)

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ABSTRACT

Monosodium glutamate (MSG) is a flavor enhancer and food additive with a unique umami taste. Due to its widespread use in humans, this study focused on the cytotoxicity, anti-diabetic effect, and interaction with protein digestion by performing a standardized static in vitro digestion model and lipid digestion by estimating free fatty acids released from 0.5 g of olive oil during intestinal lipolysis. The study showed that monosodium glutamate has an apparent cytotoxic effect on the Caco-2 cell line in a dose-dependent manner. MSG glutamate also showed low inhibitory activity on alpha-glucosidase enzyme even at high concentrations (16.3 % at 1800 ppm). By performing simulated in vitro digestion to study the interaction between MSG and protein digestion, followed by MTT study, total protein determination, and pH drop method, all results concluded that MSG affected proteolysis. Finally, the impact of MSG on lipolysis was studied through a free fatty acid release test. The results of the study demonstrated that MSG harmed fat digestibility in a concentration-dependent manner. As a result, it is essential to conduct further studies, especially in vivo studies, to determine the potential negative effects of MSG on human health.

1 Introduction

A food additive is any substance added to processed food to improve its taste, quality, chemical properties such as alkalinity or acidity, and physical properties, including consistency, texture, and color. Moreover, food additives are used as antioxidants, flavorings and coloring agents, preservatives, sweeteners, and thickeners (Wu et al. 2021). MSG is a widely used flavor enhancer. According to European legislation, it is also known as E621 and is available in a crystalline powder form that can easily dissolve in water (Hajihasani et al. 2020). MSG's specific taste is called umami. Umami is a meaty flavor and is one of the five basic tastes besides salty, sweet, sour, and bitter (Kurihara 2015). There is considerable controversy around MSG safety. However, the European Food Safety Association (EFSA), the World Health Organization (WHO), and the Food and Drug Administration (FDA) considered MSG to be safe, with 30 mg per kilogram of body weight per day as an acceptable daily intake (ADI), although many studies revealed its toxicity and harmful effects on human health (Henry-Unaeze 2017; Zanfirescu et al. 2019). Moreover, numerous studies have linked MSG to obesity and metabolic disorders such as insulin resistance, diabetes, and high blood sugar (Araujo et al. 2017; Niaz et al. 2018). MSG could cause obesity by increasing the food's pleasant taste and disturbing the leptin-mediated hypothalamus signaling cascade (He et al. 2011). The concern is that the studies and data are inconsistent; for example, some recent studies have suggested that umami taste reduces hunger's post-ingestive recovery and suppresses obesity (Stańska and Krzeski 2016).

Previous studies have demonstrated inconsistent results regarding MSG's impact on brain health. The concern about the effect of MSG on brain health was raised because glutamate in MSG and the body are identical, and it is crucial in brain function as an essential neurotransmitter (Kazmi et al. 2017; Chakraborty 2019). Many studies have reported that MSG is neurotoxic at neonatal administration and hypothalamus neurons destruction in rats, which causes metabolic abnormalities such as pseudo-obesity, growth disturbances, depressive-like behaviors, hypogonadism, metabolic dysfunctions, and chronic inflammation (Bodnár et al. 2001; Perelló et al. 2003; Rosa et al., 2015; Göbel et al. 2017). Other studies have stated that MSG can also contribute to neurodegenerative diseases such as Parkinson's and Alzheimer's (Appaiah, 2010). MSG also negatively affects rats' memory and cognitive skills. A recent study reported that MSG reduced learning capabilities and shortened memory in rats, even with low doses (Abdel Moneim et al. 2018). Further, Ali et al. (2000) reported that MSG, even at low doses, could affect cognition during early childhood, a period in which the brain is accessible and vulnerable due to the blood-brain barrier's high permeability to small and large molecules (Ali et al. 2000). Several reports have indicated that MSG is genotoxic, and reactive oxygen species (ROS) and oxidative stress are crucial in MSG-induced genotoxicity and cytotoxicity in rats' brains, kidneys, and livers (Farombi and Onyema 2006).

In an in vitro study on human peripheral blood lymphocytes, it has been reported that MSG can cause DNA damage and genotoxic effects on the exposed cells (Ataseven et al. 2016). Ismail (2012) suggested that MSG could harm male reproductive health, causing testicular harm and infertility. On the other hand, many researchers believe that MSG is safe and causes no genotoxicity (Shibata et al. 1995; Rogers 2016). Oxidative stress occurs when reactive oxygen species are elevated. It leads to lipids, carbohydrates, proteins, nucleic acid damage, cellular metabolism disruption, and apoptosis (Saeidnia and Abdollahi 2013), which is the fundamental reason for MSG hepatotoxicity. A study by Onyema et al. (2006) on rats reported that a dose of 0.6 mg/g body weight of MSG induced

hepatotoxicity and oxidative stress by decreasing reduced glutathione (GSH) level, inducing lipid peroxidation (LPO), and increasing the activities of catalase, superoxide dismutase (SOD), and glutathione-s-transferase (GST) in the liver of the rats. In addition, liver enzymes, including alanine aminotransferase (ALT), ϒ-glutamyl transferase (GGT), and aspartate aminotransferase (AST), were significantly elevated in blood serum (Onyema et al. 2006). Another study on rats by Elshafey et al. (2017) stated that exposing rats to a dose of 4 mg/kg MSG for 90 days reduced antioxidant enzymes and increased lipid peroxidation and fibrosis. Some people can experience adverse effects after consuming MSG. This condition is called the MSG symptom complex, which includes flushing, dizziness, difficulty breathing, headache, weakness, numbness, muscle tightness, and even loss of consciousness (Zanfirescu et al. 2019). According to the Federation of American Societies for Experimental Biology (FASEB) and FDA reports, some mild short-term, transient symptoms, such as tingling, headache, palpitations, numbness, flushing, and drowsiness, may happen in some sensitive people who consume 3 g or more of MSG without food (Food & Administration, 2012). This study aimed to determine the MSG's physicochemical properties, cytotoxic effect on the Caco-2 cell line, and antioxidant behavior and to assess its interaction with the digestion process using a standardized in vitro static digestion model.

2 Materials and Methods

2.1 Chemicals and reagents

During this study, 0.05% Trypsin-EDTA (Himedia, India), Dulbecco's modified Eagle's medium (DMEM) (Himedia, India), DMSO (Lobalohemia, India), Phosphate buffered saline (PBS) (Serox, Germany), Fetal bovine serum (FBS) (Biochrom), DPPH (Himedia, India), methanol (Elnasr, Egypt), NaCl (Supelce, Germany), $MgCl_2 \cdot 6H_2O$ (LOBA, India), $(NH_4)_2CO3$(LOBA, India), KCl (Supelco, Germany), KH_2PO_4 (Supelco, Germany), $NaHCO_3$ (Supelco, Germany), MSG (Loba Chemie, India), HCl Hydrochloric acid (2N) (Samchun chemicals), Sodium hydroxide (Carl Roth), pepsin (Loba Chemie, India), pancreatin (LOBA Chemie, India), $CaCl_2 \cdot H_2O$ (Chem lobnu Belgium), and bile (LOBA Chemie, India) were used without additional purification. Caco-2 cell lines were collected from Vacsera, Giza, Egypt. Powdered milk, olive oil, and hen eggs were purchased from the local market, and whey protein isolates were obtained from a gym supplement shop. Olive oil was used for free fatty acid release tests.

2.2 Physiochemical characterization

X'Pert PRO-PAN analytical diffractometer was used to perform X-ray diffraction (XRD) measurements, with Cu-Kα radiation (λ= 1.54056 A˚) at 40 kV and 30 mA to examine the polycrystalline nature of MSG to determine crystallites size using the Scherer equation (Mele et al. 2022).

$$D = \frac{0.9\lambda}{\beta \cos\theta} \quad (1)$$

Where λ is the wavelength in A˚, *K* is the shape constant (~ 0.9), β is the observed peak width at half-maximum height in red, θ is the Bragg angle in degrees, and D is the crystallite diameter in A˚. The functional groups were pinpointed using FTIR-4100 type A in the 349.053 -7800.65 cm^{-1} range, with a resolution of 4 cm^{-1} at room temperature.

2.3 Cytotoxicity of MSG (MTT assay)

The MTT assay was performed using Caco-2 cells according to the Van Meerloo protocol (Van Meerloo et al. 2011). In brief, MSG was dissolved in water with different concentrations (200, 100, 50, 25, and 12.5 mM). Caco-2 cells were cultured at 37 °C and 5% CO_2 for 24 h, then exposed to different concentrations of MSG solutions. After 24 hrs of exposure, PBS was used to wash the cells, and 50 µL of MTT solution and 50 µL of serum-free media were added into each well. Finally, after finalizing the procedure, the absorbance was measured at a wavelength of 590 nm. The values were calculated three times and the means of three replicates were used as the final results. The percent cytotoxicity was calculated by equation 2 (Van Meerloo et al. 2011):

$$\text{Cell viability\%} = \frac{\text{Mean OD treated well } [-\text{blank}]}{\text{Mean OD control well } [-\text{blank}]} \times 100 \quad (2)$$

Where OD is the optical density.

2.4 Antioxidant activity of MSG

2.4.1 DPPH assay

Antioxidant activity was determined by the DPPH assay (Boly et al. 2016). By dissolving in water, five different MSG solution concentrations were prepared; 20, 16, 12, 8, 4, and 2 g/mL. Finally, color intensity was measured at 517 nm UV-visible light using a spectrophotometer (SPECORD 200 PLUS, Analytik Jena, Germany). The data were obtained by equation 3 and represented as means ± SD.

$$\text{RSA \%} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100 \quad (3)$$

Where RSA is radical scavenging activity, and Abs is absorbance.

2.4.2 Iron chelation assay

The assay was carried out according to the method of Santos et al. (2017), with minor modifications. In 96 well plates, 50 µL of the

sample (MSG solution with a final concentration of 5000 µg/mL) was mixed with 20 µL of the freshly prepared ferrous sulfate (0.3 mM). At the end of the incubation period, the intensity of the produced color was measured at 562 nm. The values were represented as means ± SD according to equation 4:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Blank}}} \times 100 \qquad (4)$$

The results were recorded using a FluoStar Omega microplate reader.

2.4.3 ABTS assay

The ABTS assay was performed according to the method of Arnao (2000) with minor modifications. A solution of MSG dissolved in water was prepared at a 100 μg/mL final concentration. At the end of incubation time, which was 30 min in the dark at room temperature, the change in color intensity of ABTS was measured at 734 nm, and the values were represented as means ± SD according to equation 5:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{Blank}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Blank}}} \times 100 \qquad (5)$$

Where Abs is the absorbance.

The results were recorded using a FluoStar Omega microplate reader.

2.5 The effect of MSG on carbohydrates digestion

2.5.1 Anti-diabetic activity assay (α -Glucosidase inhibition)

To evaluate the anti-diabetic activity of MSG, an α-Glucosidase inhibition assay was performed as described in previous studies (Elya et al., 2012; Qaisar et al. 2014). Finally, α-Glucosidase activity was calculated using a spectrophotometer at 405 nm by measuring the released yellow p-nitrophenol quantity. The MSG sample concentration ranged between 1800 and 23.8 µg/mL and the positive control was acarbose. The blank replaced the enzyme by adding nitrophenyl α-D-glucopyranoside with buffer solution instead. The inhibitory activity was expressed as percentage inhibition (%) using equation 6:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100 \qquad (6)$$

2.6 The effect of MSG on protein digestibility

2.6.1 In vitro digestion model

This study used the standardized static in vitro digestion model (Minekus et al. 2014). Three different phases of digestion were stimulated in the in vitro digestion models: the salivary, gastric, and intestinal phases. Each phase had different enzyme compositions and electrolytes, simulated as described in the protocol of Minekus et al. (2014).

To assess the behavior and interactions of MSG with protein digestion, two digested samples were prepared, and among these, one contained egg white protein only, while the other contained egg white protein with MSG. Then, the digested samples were taken to investigate protein digestibility in both samples using SDS-PAGE and total protein determination.

2.6.2 SDS-PAGE

SDS–PAGE was performed on the digested samples at the Unit of Analytical Chemistry, Faculty of Science, Assiut University, Egypt, using Biometra according to Laemmli's procedure (LAEMMLI, 1970).

2.6.3 Total protein determination

Total protein determination was performed for samples at the Unit of Analytical Chemistry, Faculty of Science, Assiut University, by the Bradford method (Kruger, 2009), using Spector UV-VIS double beam PC scanning spectrophotometer UVD-2950 Labomed.

2.6.4 pH drop method

The method of pH drop was performed as described by Hsu et al. (1977). 50 mL of protein solutions (6.25 mg protein source/mL) were prepared and adjusted to pH 8. A pancreatin solution was also prepared and adjusted to pH 8, and the protein solution was stirred at 37 °C. 5 mL of the solution was dropped on the protein solution, and the pH drop was recorded each minute for 10 minutes. For this regression, equation (7) was used**.**

$$Y = 210.464 - 18.103X \qquad (7)$$

Where Y is protein digestibility %, and X is the final pH at 10 minutes of digestion in the multi-enzymatic medium. The procedure was repeated for each protein source alone, and protein source besides adding 1 g and 2 g MSG; all values are means of triplicated records.

2.7 Fats digestibility

2.7.1 Fat digestibility (Free fatty acids release %)

The digestion activity of lipase for fats was measured by determining the release of free fatty acids from 0.5 g of olive oil during 30 minutes of lipolysis using a titration method mentioned in previous studies (Ji et al. 2019; Li et al. 2011). A lipase solution was prepared by dissolving 500 mg of lipase powder in 50 mL of simulated intestinal fluid, and a final concentration of 10 mg/mL

was prepared under stirring at 37 °C. Then, 100 µL of bile salt solution (160 mM) and 20 µL of $CaCl_2$ were added to 5 mL of previously prepared simulated intestinal fluid solution containing lipase enzyme while stirring. Furthermore, 0.5 g of olive oil was added to the solution. The mixture was left under stirring for 5, 10, 20, and 30 minutes. At the end of lipolysis, 10 mL of (95%) ethanol was added to the mixture to stop lipase enzyme activity, and 1% (w/v) phenolphthalein was used as an indicator. A direct titration with 0.1 N NaOH to a phenolphthalein endpoint was performed using a burette. All the mentioned steps were repeated by adding 50 mg and 100 mg of MSG to the 5 mL of simulated intestinal fluid containing lipase. The FFA% release was calculated using the equation reported in previous studies (Li and McClements 2010).

2.8 Statistical analysis

The data were represented as mean±SD. The data were analyzed using *Microsoft Excel*®, and the IC_{50} value was calculated using *Graphpad Prism 6*® by converting the concentrations to their logarithmic value and selecting a non-linear inhibitor regression equation (log (inhibitor) vs. normalized response – variable slope equation).

3 Results and discussion

3.1 Physiochemical properties of MSG

3.1.1 XRD analysis

The XRD pattern of MSG is presented in Figure 1. The diffraction pattern reflected the polycrystalline nature of MSG with a characteristic peak at 2θ (10.018, 20.027, 25.525, 38.241, 46.474, and 51.380), which agreed with the XRD reported by Saeidnia and Abdollahi (2013). The calculated crystal size of MSG from the Scherer equation was 40.13 ± 12.6 nm, which reflected the nano nature of MSG. This smaller size might be responsible for the cytotoxic effect of MSG as it facilitated its uptake through cells.

3.1.2 FTIR spectrum of MSG

The FTIR spectra of MSG were characterized by several vibrational bands, as shown in Figure 2. The vibrational bands observed in the 3000 ~ 3600 cm^{-1} corresponded to O-H stretching vibration in the molecule, which was formed due to hydrogen bonds. The vibrational band observed at 2900 cm^{-1} was due to C-H stretching vibration, while C=O stretching vibration appeared at 1687 cm^{-1}. The band observed at 1604 cm^{-1} was due to N-H stretching vibration. The bands at 1528 cm^{-1} and 1404 cm^{-1} correspond to C=C and –COO stretching vibrations, respectively. The absorption bands at 1104, 613, and 524 cm^{-1} were attributed to the stretching vibration of -COOH, wagging vibration of $(COO)^-$ and the deformation of HOCC, respectively (Onyema et al. 2006).

3.2 Cytotoxicity of MSG (MTT assay)

The number of viable cells of Caco-2 cells after exposure to different concentrations of MSG (12.5, 25, 50, 100, and 200 mM) was estimated by the MTT assay. Figure 3 demonstrates the cell viability curve as estimated from the MTT assay. A gradual decrease in the cell viability was observed from 136% to 90% as the concentration of MSG increased from 12.5 to 50 mM. However, at the higher concentrations, a slight decrease in the cell viability from 90% to 81%, with an increase in MSG concentration from 50 to 200 mM, was reported. These results revealed the cytotoxic effect of MSG, which was consistent with many previous studies (Elshafey et al. 2017). However, a few studies showed that MSG was not cytotoxic on RGA and H295R cell lines (Shannon et al. 2019).

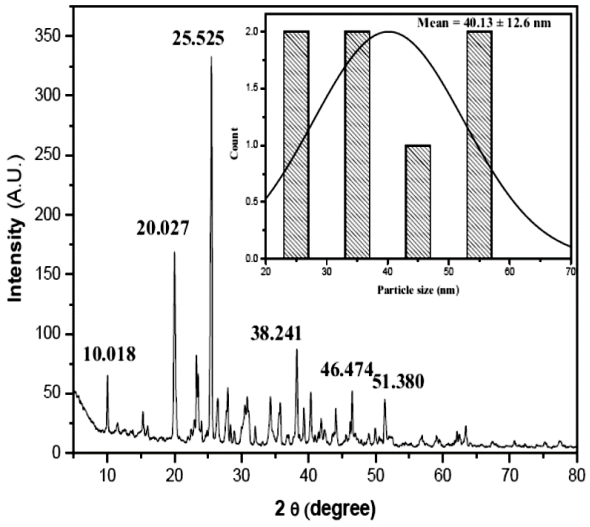

Figure 1 XRD pattern of MSG.

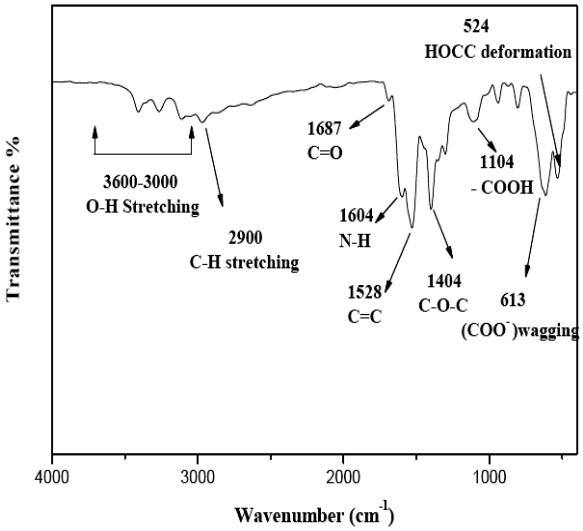

Figure 2 FTIR pattern of MSG.

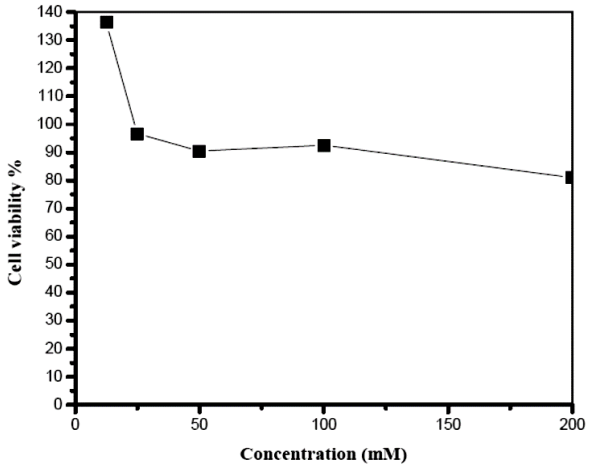

Figure 3 Cell viability of Caco-2 cell line after exposure to MSG with different concentrations (200, 100, 50, 25, and 12.5 mM).

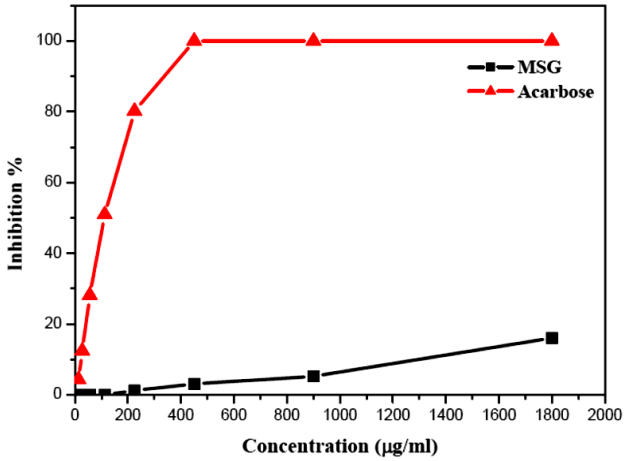

Figure 4 The alpha-glucosidase inhibition rate of MSG compared to acarbose.

Table 1 The inhibition rate of alpha-glucosidase by MSG compared to acarbose

Sample	LC_{50} (µg/mL)	LC_{90} (µg/mL)	Inhibition%
MSG	-------	-------	16.3% at 1800ppm
Acarbose	125±3	245±2.7	100% at 1800ppm

Table 2 Total protein content in the digested egg white protein only and egg white protein with 1 gm of MSG after the whole digestion process

Egg white protein only	5100 mg/l
Egg white protein + MSG	4349 mg/l

3.3 Antioxidant activity of MSG

3.3.1 DPPH assay

No action was detected, which meant it had no scavenging activity.

3.3.2 Iron chelation assay

No action was detected, which meant it had no iron chelation activity.

3.3.3 ABTS assay

No action was detected even with escalating the MSG concentration up to 100 µg/mL, assuring that MSG has no scavenging activity.

3.4 The effect of MSG on carbohydrate digestibility

3.4.1 Alpha-Glucosidase inhibition - anti-diabetic activity assay

The alpha-glucosidase inhibition assay in Figure 4 showed that MSG has a low inhibition effect on the alpha-glucosidase enzyme, even at the highest concentration. As illustrated in Table 1, the inhibition percentage reached only 16.3% by MSG at 1800 ppm, while in the case of acarbose, which served as a standard, it reached 100% inhibition at 450 ppm; the IC_{50} of acarbose was 125±3 ppm, and IC_{90} was 245±2.7 ppm.

3.5 The effect of MSG on protein digestibility

3.5.1 SDS-PAGE

SDS-PAGE was performed to assess the in vitro digestion of egg white protein only and egg white protein with MSG. As shown in Figure 5, all unique bands of egg white protein digested samples disappeared, which revealed its susceptibility to proteolytic enzymes in gastric and intestinal phases. A light aggregation was detected in the case of egg white protein with MSG-digested samples at 17 kDa, likely for lysozyme (Wang et al. 2018). Our results showed that MSG could alter protein digestibility.

3.5.2 Total protein determination

We applied the Bradford method for protein determination in digested egg white protein samples with and without MSG addition. Table 2 represents the results of total protein content after digestion. The digested protein content in the sample containing only digested egg white protein was 5100 mg/L, which was significantly higher than the digested protein content in the egg white protein with MSG digested sample (4340 mg/L). Our results suggest

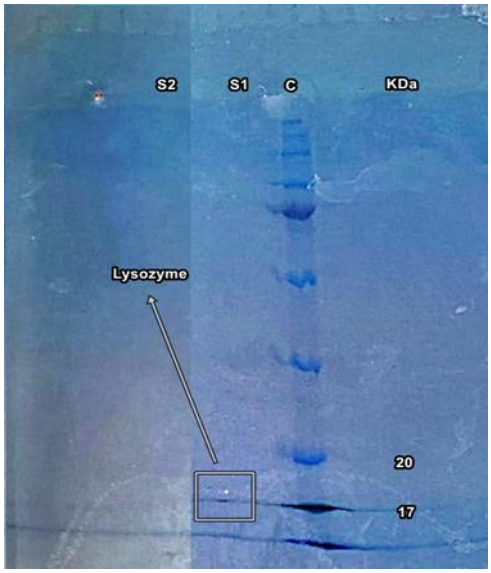

Figure 5 SDS-PAGE for digested egg white protein only (S2) and digested egg white protein with MSG (S1). The markers of standard Mw are in the right lane (c).

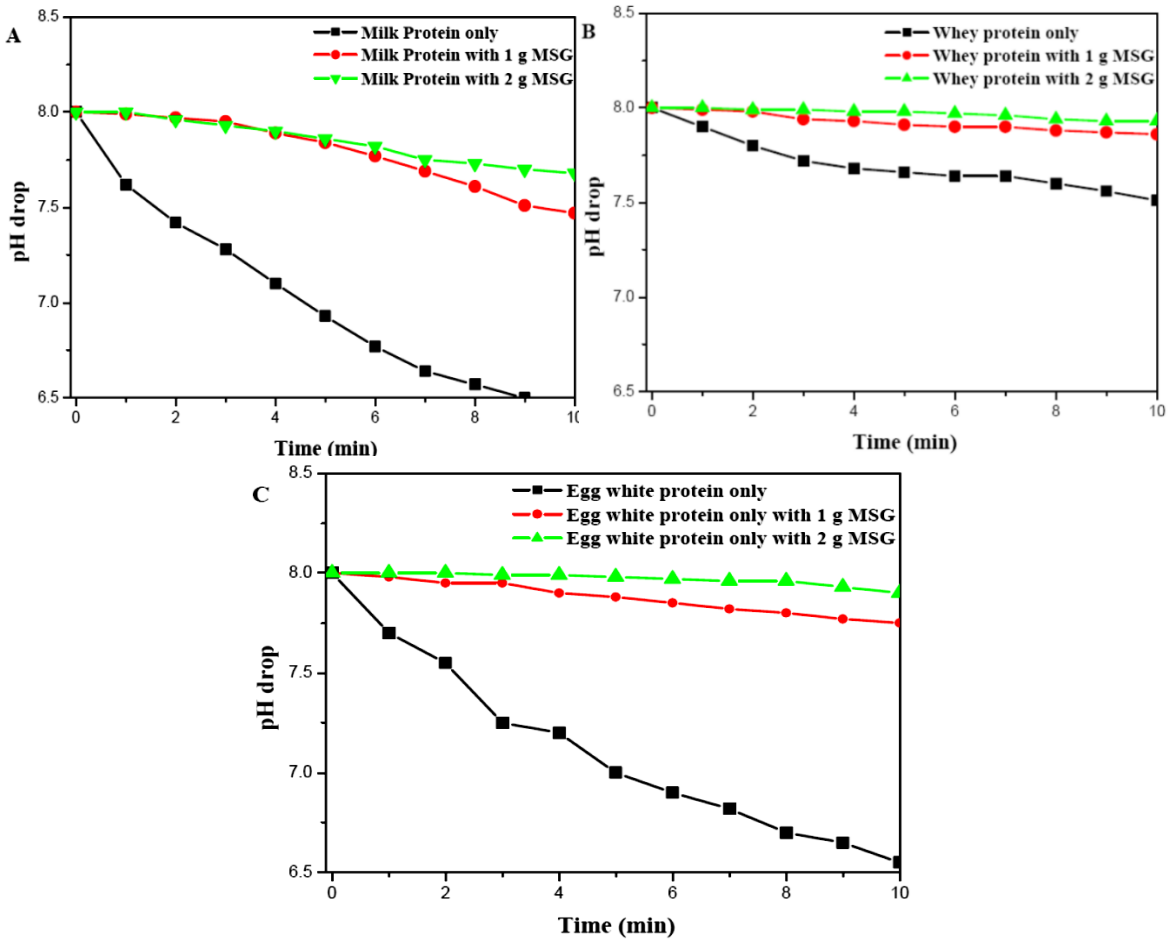

Figure 6 pH drop curves over 10 minutes of digestion with different concentrations of MSG (1 and 2 g) for powdered milk protein (A), whey protein isolate (B), and egg white protein only and with the addition of 1 g and 2 g MSG (C).

that MSG suppresses protein digestion while no previous studies have dealt with this effect to the best of our knowledge.

3.5.3 pH drop method and protein digestibility

Figure 6 describes the pH drop method for the three types of proteins used in this study in the presence of pancreatic supports protein *in-vitro* digestion results were mentioned before. It was observed that protein digestibility was suppressed by adding 1 g and 2 g MSG to each protein solution during digestion in a dose-dependent manner. In addition, Figure 7 represents the protein digestibility as calculated from regression equation 6. The protein digestibility of powdered milk proteins after 10 minutes of digestion was 93.89%, decreased to 75.25 % with adding 1 g MSG and dropped to 71.45% with adding 2 g MSG. Whey protein showed low digestibility. In the case of whey protein isolate alone, protein digestibility after 10 minutes of digestion was 74.53. Its digestibility decreased to 68.19% with adding 1 g MSG and dropped to 66.93% with adding 2 g MSG. Egg white protein digestion after 10 minutes of digestion was 91.91%. Its digestibility decreased to 70.19% with adding 1 g MSG and dropped to 67.47% with adding 2 g MSG.

3.6 The effect of MSG on fat digestibility (FFA release %)

The FFA of percentage release represented in Figure 8 showed a negative effect of MSG presence in the intestinal phase digestion of lipids over 30 minutes in a dose-dependent manner. The olive oil digestion FFA release percentage reached 41.47%. FFA percentage release decreased by adding 50 mg of MSG to 27.64%

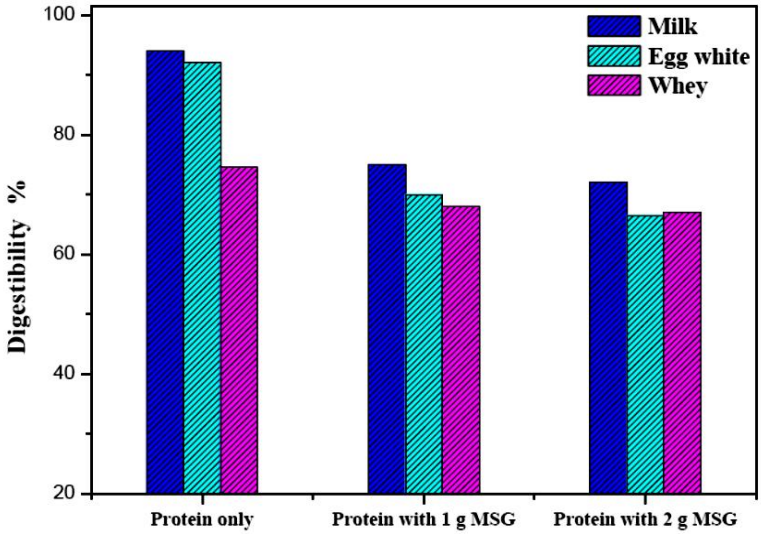

Figure 7 Protein digestibility of powdered milk protein, egg white protein, and whey protein isolate alone and in addition of 1 and 2 g MSG.

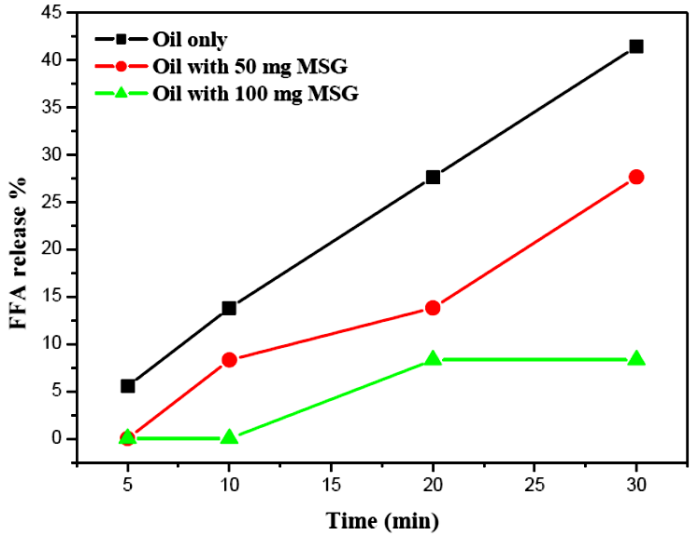

Figure 8 FFA releases % from 0.5 mg of olive oil with and without adding MSG.

and by 8.34% when adding 100 mg of MSG to the digestion media. The current results are novel due to the lack of previous studies concerned about the inhibitory effect of MSG on intestinal lipolysis. To the best of our knowledge, we found only an in vivo study that hypothesized that MSG might alter the lipid lipolysis processes in the small intestine's lumen (Kohan et al. 2016). Another study suggested that MSG administration in rats caused a significant decrease in lipolysis but not in adipose tissue (Dolnikoff et al. 2001).

Conclusion

MSG is a ubiquitous food additive categorized as a flavor enhancer. This study shows that MSG can have a cytotoxic effect on the Caco-2 cell line. MSG's antioxidant activity demonstrates that it has no antioxidant activity at all. In contrast, it leads to oxidative stress through reactive oxygen species (ROS). MSG has a very low inhibition effect on one of the main enzymes contributing to carbohydrate metabolism, alpha-glucosidase. MSG also negatively affects protein and lipid digestion in the gastrointestinal tract (GIT). This study recommends and stresses the significance of more investigations into MSG's interaction with digestive enzymes and nutrient digestion to declare its safety.

Authors' contributions

Alaa Hassan Said wrote the original manuscript and analyzed the data. M. Yasser Alsedfy did the experimental part. A.A. Ebnalwaled and Mona Moustafa revised the original manuscript. The original manuscript was approved and revised by all authors.

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Not applicable.

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