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EFFECTS OF SODIUM OCTANOATE, ACYLATED GHRELIN, AND DESACYLATED GHRELIN ON THE GROWTH OF GENETICALLY ENGINEERED *ESCHERICHIA COLI*

UTICAJ NATRIJUM-OKTANOATA, ACILIRANOG GRELINA I DEACILIRANOG GRELINA NA RAST GENETIČKI STVORENE *ESCHERICHIAE COLI*

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Summary: Acylated ghrelin is a 28-amino acid peptide hormone bearing a fatty acid group based on octanoic acid (caprylic acid) at the serine which is located at position 3 and at the N-terminus. If this fatty acid is cleaved from acylated ghrelin, the remaining peptide is referred to as desacylated ghrelin. Free fatty acids (FFAs) can kill or inhibit the growth of bacteria. The purpose of this study was to test this ability using acylated ghrelin, desacylated ghrelin, and sodium octanoate (caprylic acid) as carbon sources for the genetically engineered *Escherichia coli* strains MK79 and MK57. For this experimental work, minimal medium was modified by replacing glucose with equal concentrations of acylated ghrelin, desacylated ghrelin, or sodium octanoate. Bacterial optical density, viability, alpha-amylase production, plasmid stability and pH of the growth medium were measured during these experiments. The media that allowed most growth, based on viable cell counts and the OD_{600} of MK79, was minimal medium, followed by the medium containing desacylated ghrelin or acylated ghrelin, and finally the medium containing sodium octanoate. The same order was observed for MK57. Neither of the strains lost plasmids during the entire course of each experiment. There was also little change in the pH of any of the media used for both strains. These results suggest that sodium octanoate, acylated ghrelin, and desacylated ghrelin, when compared with minimal medium, inhibit *Escherichia coli* growth. Proliferation was lowest when sodium octanoate was used as the carbon

Kratak sadržaj: Acilirani grelin je hormon koji čini peptid od 28 aminokiselina koji nosi grupu masnih kiselina na bazi oktanoinske kiseline (kaprilna kiselina) u serinu i koji se nalazi na poziciji 3 i na N-terminalu. Kad se ta masna kiselina odvoji od aciliranog grelina, preostali peptid se naziva deacilirani grelin. Slobodne masne kiselina (FFAs) mogu uništiti ili sprečiti rast bakterija. Cilj ove studije bio je da se ispita ta njihova sposobnost korišćenjem aciliranog grelina, deaciliranog grelina i natrijum-oktanoata (kaprilne kiseline) kao izvora ugljenika za lance *Escherichiae coli* MK79 i MK57 stvorene genetskom manipulacijom. Za ovaj eksperimentalni rad, minimalni medijum modifikovan je zamenjivanjem glukoze jednakim koncentracijama aciliranog grelina, deaciliranog grelina ili natrijum-oktanoata. Tokom eksperimenta su mereni optička gustina, vijabilnost, produkcija alfa-amilaze, stabilnost plazmida i pH medijuma za rast. Medijum koji je omogućio najveći rast, na osnovu brojenja vijabilnih ćelija i OD600 MK79, bio je minimalni medijum, sledi medijum sa deaciliranim grelinom ili aciliranim grelinom, i najzad medijum sa natrijum-oktanoatom. Isti redosled uočen je kod MK57. Nijedan od lanaca nije gubio plazmide za vreme čitavog toka svakog eksperimenta. Takođe, gotovo da nije bilo promena pH u medijima korišćenim za oba lanca. Naši rezultati ukazuju na to da natrijum-oktanoat, acilirani grelin i deacilirani grelin u poređenju sa minimalnim medijumom sprečavaju rast *Escherichiae coli*. Proliferacije je bila najniža kada je kao izvor ugljenika korišćen natrijum-oktanoat, zatim acilirani grelin

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source, followed by acylated ghrelin and desacylated ghrelin. Therefore, the acylated ghrelin found previously in human saliva might help to inhibit pathogenic microorganisms, and acylated ghrelin levels below a critical concentration in saliva could result in an increased risk of oral infection.

Keywords: sodium octanoate, *Escherichia coli*, acylated and desacylated ghrelin, growth, alpha-amylase

pa deacilirani grelin. Dakle, acilirani grelin prethodno nađen u ljudskoj pljuvački mogao bi doprineti inhibiciji patogenih mikroorganizama, dok bi nivoi aciliranog grelina ispod kritične koncentracije u pljuvački za posledicu mogli imati povećan rizik od oralne infekcije.

Ključne reči: natrijum-oktanoat, *Escherichia coli*, acilirani i deacilirani grelin, rast, alfa-amilaza

Introduction

Ghrelin is a 28-amino acid lipopeptide-appetite hormone that stimulates the secretion of growth hormone from the anterior pituitary gland. Ghrelin has multiple effects other than on growth hormone secretion, including gastrointestinal coordination, energy metabolism, facilitation of cell survival and proliferation, inhibition of apoptosis, and regulation of food intake (1, 2). Ghrelin was originally isolated from rat stomach and is produced mainly by endocrine X/A-like cells in the gastric mucosa of rats (3). It is also produced by P/D1 cells lining the fundus of the stomach in humans. In addition to the major organ in which it is synthesized, the stomach, it is also widely expressed in kidney, heart, lung, liver, brain, adipose tissue, adrenal glands, and other parts of the gastrointestinal tract (1, 2). Its mRNA is ubiquitous (4). Ghrelin has two main forms. Both forms exist in biological fluids such as serum, plasma, saliva, and milk and in tissues (1, 5). One of its forms, called »acylated ghrelin«, is acylated in position 3 and has an octanoyl (caprylic acid: octanoate) group at the N-terminal serine. This molecule is the first known example of a peptide hormone bearing a fatty acid. If the fatty acid (octanoate) is cleaved from the peptide, the resultant fatty acid-free ghrelin is »desacylated ghrelin« (3). Lactone is present in many species of bacteria and is an example of the cell-cell signaling molecules. Structural homology between this molecule and acylated ghrelin (O-acylation of serine) could be due to evolutionary convergence (6).

Utilization of long-chain fatty acids is widespread among microorganisms (e.g., *Pseudomonads*, *Bacilli*, *Escherichia coli*), and *E. coli* takes up palmitic acids from the environment faster than any other bacteria does (7). These fatty acids are degraded by β-oxidation. Free fatty acids (FFAs) can kill or inhibit the growth of bacteria (8, 9). For example, caprylic acid and monocaprylin have antibacterial effects on mastitis pathogens (10) and also inactivate bacterial fish pathogens (11), as do medium-chain lipid molecules (caprylic acid, onocaprylin and sodium caprylate). Acylated ghrelin, in addition to its 28 amino acids, contains octanoic acid (8 carbons). From these biochemical properties it has been proposed that both free octanoic acid (octanoate) and acylated ghrelin, which contains octanoic acid, might be suitable as carbon sources or can be inhibitory for *E. coli*. When the fatty acid (octanoate) is cleaved from the peptide, 28-amino acid residue (fatty acid-free ghrelin) is desacylated ghrelin. Amino acids and low molecular weight peptides yielded by proteolysis are actively taken up and used for growth by many microorganisms (12–14). However, the biochemical properties of these carbon sources have not yet been investigated, and this is the purpose of our study.

Thus, two genetically engineered *E. coli* strains were used for tests; one had only the *Bacillus stearothermophilus* alpha-amylase gene (MK57) and the other the same gene plus the VHb gene (*vgb*) (MK79). In testing the suitability of sodium octanoate, acylated and desacylated ghrelin as carbon sources, and also their effect on microbial growth, optical density, alpha-amylase production, viability, plasmid stability and the pH of the growth medium were measured (15).

Material and Methods

Chemical, Hormones and Enzymes

Acylated ghrelin (catalog no. 51515) and desacylated ghrelin (catalog no. 88142) were obtained from GL Biochem. Sodium octanoate (catalog. no: C5038-100G) was obtained from Sigma-Aldrich.

Bacterial strains and Plasmids

Two genetically engineered *E. coli* strains (MK57 and MK79) were used. MK57 was constructed by transforming the *E. coli* strain JM103 with the plasmid pMK57. MK79 was constructed by transforming the *E. coli* strain JM103 with the plasmid pMK79. The plasmid pMK79 was created by cloning a 3 kb fragment containing the *Bacillus stearothermophilus* alpha-amylase gene into the plasmid vector pUC8. Later, a 2.3 kb segment of *Vitreoscilla* DNA containing *vgb* was inserted into pMK57 to create pMK79. Before starting the experiments, recombinant strains were selected in the presence of 100 μg/mL ampicillin (amp), and the presence of each plasmid was confirmed by miniprep DNA analysis (data not shown). These genetically engineered ampicillin-resistant strains were chosen to eliminate possible contamination.

Growth Media

Minimal medium (16) contained KH2PO4 (22 mmol/L); $NaHPO_4$ (49 mmol/L); NH_4Cl (19 mmol/L); $MgSO_4$ (2 mmol/L) and glucose (11 mmol/L). The pH values of all media were adjusted (with 6 mol/L sodium hydroxide) to 7.0 at room temperature.

To test the effects of sodium octanoate, acylated ghrelin, and desacylated ghrelin on *E. coli* growth and on the recombinant protein (alpha amylase), minimal medium was modified by replacing the glucose with acylated ghrelin, desacylated ghrelin, or caprylic acid. All other media components remained the same.

For each experiment, a single colony from a plate was inoculated into 5 mL LB-amp medium in a 25 mL flask, which was then incubated at 37 °C overnight on a shaker at 125 rpm. From each culture, 100 µL was then transferred into 4.9 mL of fresh LB-amp medium in a 25 mL flask and incubated under the same conditions for 4 h. At 4 h intervals thereafter, 1 mL of culture was put into a microfuge tube and spun for 5 min. The supernatant was removed and the cell pellet washed twice with phosphate buffer. The initial OD_{600} reading of the washed cell suspension was adjusted with phosphate buffer to an OD_{600} of 0.02, from which 100 µL were inoculated into shaker flasks holding 4.9 mL of minimal medium or modified minimal medium, and incubated for 24 h or 48 h.

The number of viable cells was determined by plating on LB following serial dilution with 0.85% NaCl. Colonies were counted after 16 h growth at 37 °C. Subsequently, 20 colonies randomly selected from the LB plates were transferred to the LB-amp plates using sterile toothpicks, and the plates were incubated under the same conditions. The colonies that grew on LB plates but not on LB-amp plates were colonies comprised of cells that had lost plasmids, thereby determining the strain's plasmid stability.

Growth of the cells was also measured spectrophotometrically by following the optical density (OD_{600}) of the culture. Samples were diluted as required with minimal medium or modified medium to maintain the OD_{600} below 0.500, and their absorbances were measured against a blank of minimal medium or the appropriate modified minimal medium. The pH of the medium was measured using a Jenway 3010 pH meter, as described previously (17).

Alpha-amylase activity assay

For alpha-amylase measurements, 1 mL samples of 24 h or 48 h cultures were centrifuged at 14,000 g for 5 min. The supernatant and pellet were separated and kept at –20 °C until they were analyzed. Alpha-amylase activities in both pellet and supernatant were determined by a spectrophotometric assay. The amounts of enzyme in both pellet and supernatant were determined as described by Liu et al. (17).

Results

For all experimental conditions, neither of the strains lost plasmid, giving a 100% plasmid stability during the entire course of each run. This result is in contrast to those of Lavastida et al. (18), who reported that, with time, plasmids became unstable inside host cells. Initially the pH of the growth medium was 7.0 in both strains and in all the growth media, although media in which MK79 were grown had a marginally lower pH than media in which MK57 were grown (*Figure 1*).

Cells of the MK79 and MK57 strains in minimal medium had a higher cell density, representing more viable cells than cells grown in the modified media (*Figures 2, 3*). However, MK79 strains grown in minimal medium grew to a lower cell density or had fewer viable cells than MK57 (*Figure 2* and *3*). The highest level of alpha-amylase activity for both strains occurred in minimal medium, followed by media containing desacylated ghrelin, acylated ghrelin, and sodium octanoate (*Figure 4*). Accumulation of the recombinant alpha-amylase protein in MK79 was less than the accumulation in MK57 cells grown in minimal medium (*Figure 4*). Overall, the medium in which glucose was the carbon source also supported high alpha-amylase activities, high cell densities, and high viable cell counts. The order of the media that supported these activities best was, from highest to lowest, minimal medium, then the media containing desacylated ghrelin, acylated ghrelin, and sodium octanoate.

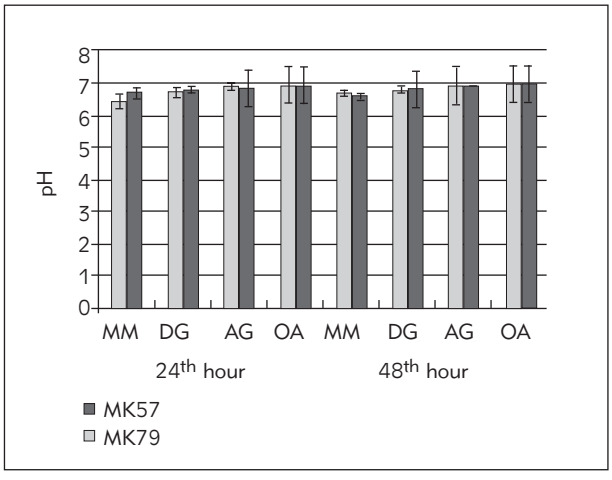

Figure 1 Change in pH of growth medium during experiments. Values are the average of three individual trials. MM, minimal medium; DG, desacylated ghrelin; AG, acylated ghrelin; OA, octanoic acid (sodium octanoate).

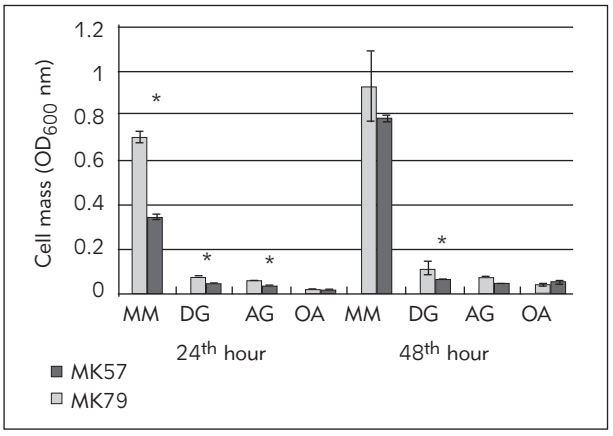

Figure 2 Growth (OD_{600}) of MK79 and MK57 (see more details under *Figure 1*). Values are the average of three individual trials.

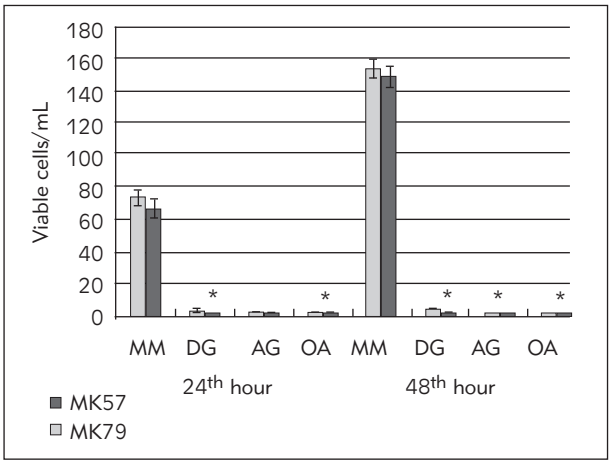

Figure 3 Growth (viable cells 10^7/mL) of MK79 and MK57 (see more details under *Figure 1*). Values are the average of three individual trials.

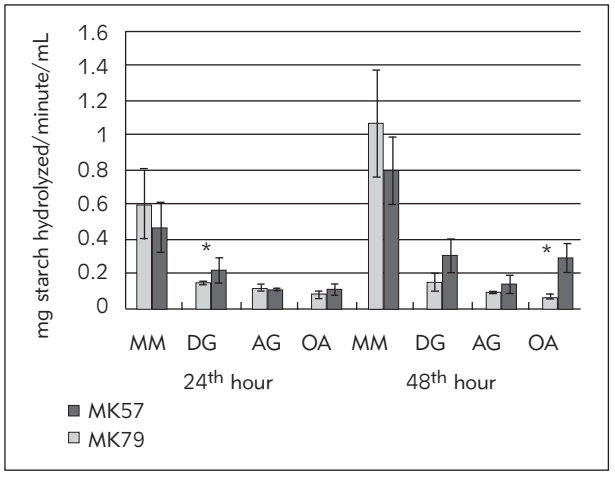

Figure 4 Effect of different carbon sources (acylated and desacylated ghrelin and octanoic acid) on the expression of alpha-amylase in *MK79* and *MK57* compared to minimal medium (see more details under Figure 1). Values are the average of three individual trials.

Discussion

Glucose and fatty acids are mainly metabolized via glycolysis and the tricarboxylic acid cycle. *E. coli* can utilize long-chain fatty acids, including palmitic acids, which are degraded by β-oxidation. Palmitic acid is first converted to its corresponding CoA ester by acyl-CoA synthetase. The CoA ester is then oxidized by β-oxidation and subsequently cleaved to produce the acetyl-CoA and CoA esters of the fatty acid shortened by 2 carbon atoms (7). One might therefore expect that the eight carbons in the even-numbered fatty acids (e.g. octanoate) present in ghrelin might be utilized by a similar pathway in the *E.* coli MK79 and MK57 strains. The data presented here do not support this assumption because the growth of both strains was dramatically inhibited by the presence of either free sodium octanoate (9–11) or acylated ghrelin, the peptide characterized by the presence of an *n*-octanoylation on the hydroxy group of the serine in position 3. Thus, our results lend credence to those of Chorny et al. (20).

The growth of the two strains might be a result of desacylated ghrelin utilization rather than the acylated ghrelin and sodium octanoate effect. The growth of both strains was almost totally inhibited by the presence of sodium octanoate, and it was similarly inhibited in the presence of acylated ghrelin. When acylated ghrelin is broken down by autolysis, free octanoic acid is released into the medium. Free octanoic acid might itself inhibit bacterial growth (9–11). Therefore, strains should grow better in the presence of desacylated ghrelin than in a medium containing acylated ghrelin. Desacylated ghrelin contains 28 amino acid residues after the octanoic acid is cleaved off. Within these 28 residues, some amino acids are not represented: asparagine or aspartic acid, cysteine, isoleucine, methionine, tyrosine, valine, tryptophan, and threonine. The rest of the amino acids, which are represented in acylated and desacylated ghrelin, might be utilized by MK79 and MK57. Amino acids and low molecular weight peptides arising from autolytic proteases are actively taken up and used for growth by a number of bacteria (12, 13). One reaction used to initiate the breakdown of amino acids is deamination. Amino acids such as serine and histidine are subject to deamination. Serine, thus treated, produces pyruvate, which is further metabolized by glycolysis. The aerobic breakdown of aromatic amino acids (e.g., histidine) also occurs in many microorganisms (7). Since MK79 and MK57 grow on modified minimal medium with desacylated ghrelin, they can clearly utilize amino acids (e.g., serine and histidine, which are present in desacylated ghrelin) that may be released by autolytic degradation of peptides.

MK79 produces less extracellular and intracellular alpha-amylase than MK57 does in minimal medium, which contains glucose as the only carbon

source. This difference was observed both per mL of culture and per OD_{600} of cells. The productivity of recombinant *E. coli* can be improved by expression of the bacterial hemoglobin gene (17–19). Decreased productivity of MK79 may be related to the decreased expression of hemoglobin due to the presence of glucose in the growth medium. It has been shown by carbon monoxide difference spectral analysis that hemoglobin expression is decreased in a medium that contains glucose (17). Plasmid-bearing cells have growth disadvantages compared with their untransformed counterparts. Glucose suppression of hemoglobin expression in MK79 might be an extra metabolic burden to the cells as the result of the cells having to replicate and express plasmid-encoded genes. This would explain why MK57 produces more amylase than MK79 in the presence of glucose. The data presented here support previously published studies demonstrating the close relationship between bacterial hemoglobin production and biomass yield (17–19). Hence, the production of this enzyme seems to be associated with the microorganism growth. The function of expressed hemoglobin in MK79 might be to enhance oxygen uptake by the membrane bound-respiratory apparatus, resulting in improved growth and productivity of recombinant cells.

The decrease in pH in the media varied with the growth conditions and between strains. Therefore, acid production can be affected by the carbon source used. In the presence of glucose, the strains produce more acid and more alpha-amylase compared to strains grown in the modified minimal media. However, media in which MK79 were grown did have a marginally lower pH than media in which the MK57 strain was grown. It has been reported that oxygen uptake rates are higher at pH 4.0 than at pH 7.0. Also, the function of bacterial hemoglobin produced by MK79 could be to enhance the oxygen uptake rate of the membrane-bound respiratory apparatus (20). Therefore, the pH of the medium for MK79 might be relatively low compared to the pH of the medium for MK57.

Conclusions

In conclusion, our experiments indicate that the growth of MK79 and MK57 is inhibited in media containing no glucose or sodium octanoate, acylated ghrelin, or desacylated ghrelin compared to growth of these strains in a medium containing glucose, an almost universally-used carbon source. Both strains grow better in the presence of desacylated ghrelin than in the medium containing acylated ghrelin or sodium octanoate. Almost no growth occurs in the medium containing sodium octanoate. Autolysis of acylated and desacylated ghrelin (by self-degradation) might be an important factor influencing growth under these conditions. Therefore, the results indicate that the concentration of acylated ghrelin might play an important role in avoiding infectious diseases. In this regard, acylated ghrelin can be seen as a natural antimicrobial peptide (21) that is widely distributed in all body tissues and is especially abundant in saliva and blood (5).

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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