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NITRIC OXIDE RADICAL SCAVENGING ACTIVITIES IN EFFECTIVE MICROORGANISMS (EM) AND COLLAGEN

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ABSTRACT

This study was to evaluate the nitric oxide (NO) radical scavenging activities extracts from effective microorganisms (EM) with/without collagen extracts. Collagen has a unique triple-helix structure with a repeated amino acid sequence of (Gly-X-Y)n, in which X and Y are typically Pro and Hyp. EM is various blends of common predominantly anaerobic microorganisms consisting of a culture of potentially beneficial microorganisms. Experiments were conducted in anticipation of the EM fermentation solution decomposing polymer collagen into low molecular weight collagen. It was observed that inhibition percentage values go on increasing with enhan**ce**ments in concentration of research EM and collagen extracts in the assay mixture. NO scavenging activity of the EM-A was evaluated 60.0% on 100% solution without collagen and that of the EM-B was 47.6% at same concentration. Three EM groups with collagen were shown higher radical scavenging effects at all concentration than those of EM groups without collagen. The values of inhibits 50% of the enzyme activity (IC_{50}) for the EM-A, EM-B, and EM-C were 264.6, 303.5, and 358.1 μg/ml, respectively

KEYWORDS: Collagen, Effective microorganisms (EM), nitric oxide radical scavenging.

INTRODUCTION

Effective microorganisms (EM) are various blends of common predominantly anaerobic microorganisms in a carbohydrate-rich liquid carrier substrate (molasses nutrient solution). The pseudoscientific concept of "friendly microorganisms" was developed by professor Teruo Higa, from the University of the Ryukyus in Okinawa, Japan[1] He stated in the 1980s that a combination of approximately 80 different microorganisms was capable of positively influencing decomposing organic matter such that it reverts into a "life-promoting" process. The concept has been challenged and no scientific studies support its main claims. This was acknowledged by Higa in a 1994 paper co-authored by Higa and soil microbiologist James F Parr.[2]

Various experimenters have examined the use of EM in making organic fertilizers and investigated the effects of the fermented organic fertilizer on soil fertility, crop growth, sewage treatment, and wastewater systems, not distinguishing the effects of the microorganisms in the EM treatments from the effect of the EM nutrient solution in the carrier substrate.[3-5] For example, the influence of effective microorganisms (EM), a commercially available microbial inoculant containing yeasts, fungi, bacteria and actinomycetes, was evaluated in field trials of commercially produced, irrigated vegetable crops on “organic” farms in Canterbury, New Zealand during 1994–1995, and in a laboratory incubation.[6]

The resulting effects on crop growth depend nonspecifically upon multiple factors, including effects of the introduced EM nutrient solution with microorganisms, effects of the naturally microorganism-rich bio-organic fraction in the soil, and indirect effects of microbial-synthesized metabolites.[7-9]

Microbial activity is important for a series of soil reactions and functions, including organic matter decomposition, humus formation, nutrient cycling, aggregate formation and stabilization. Production of food, the removal of pollutants, and development of a healthy environment are all determined by microbial activity. The loss of useful microbial activity in our environment coupled with geological processes and altered the chemical environment of Earth's surface. The evolution of oxygenic photosynthesis in cyanobacteria was undoubtedly the single most important step, since the origin of oxygenic photosynthesis gave rise to an O2-containing atmosphere without which multicellular organisms cannot survive.[10]

Despite many studies on EM fermentation is a substance

that helps microbial activities such as agriculture, forestry, and environmental pollution purification, few studies on collagen and fusion are being conducted. Experiments were conducted in anticipation of the EM fermentation solution decomposing polymer collagen into low molecular weight collagen. The aim of the present work was to perform a screening of the NO• scavenging activities of the EM with collagen extracts.

MATERIALS AND METHODS

Sample extract

Three EMs were purchased. The bacteria commonly contained are as Table 1. Only Lactic acid bacteria used different strains. Namely, EM-A was used *Lactobacillus plantarum* and EM-B was *Lactobacillus casei*. EM-C was used *Streptococcus lactis.*

Table 1: Major microorganisms of EM in this study.

Type of microorganisms	Main species
Lactic acid bacteria	EM-A: *Lactobacillus plantarum*
	EM-B: *Lactobacillus casei*
	EM-C: *Streptococcus lactis*
Photosynthetic bacteria	*Rhodopseudomonas palustris*
Yeasts	*Saccharomyces cerevisiae*
Actinomycetes	*Aspergillus oryzae, Mucor hiemalis*

Others contained beneficial microorganisms in nature combine into EM in the manufacturing process and survive in the mixture of EM at pH level under 3.5.

Various concentrations of EM was further mixed with 10 mg collagen powder.

Nitric oxide (-NO) scavenging assay

The procedure of NO scavenging assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent.[11] The Nitric oxide scavenging activity was measured by the method described by Marcocci et al.[12] The final volume of the reactant was 1.2 ml for 1 mM $NaNO_2$ 120 ul, 0.1 N HCl 840 ul, and various concentrations of specimens. After the reaction for an hour at 37°C, 1 ml of the reactant was mixed with 3 ml of 2% acid and 400 µl of the Griess reagent and it was reacted for 15 minutes at room temperature. The amount of nitrite remaining was measured by checking the absorbance at 520 nm using a spectrophotometer. Quercetin was used as a positive control.

All analysis was carried out at least in triplicate. The results were expressed as the mean ± SD. Significance and confidence level were estimated at $p < 0.05$.
The percent inhibition was calculated as the decolourization percentage of the test sample using the following formula:
Inhibition (%) = (IA-As)/IA×100.

Where IA is the absorbance of the 100% initial and As is the absorbance of the sample. IA and As were the values which were subtracted the average absorbance of the blank wells.

Inhibitory analysis

Data was conducted using Microsoft Excel and SPSS 21.0 for Windows (Chicago, IL, USA). A one-way and a two-way analysis of variance (ANOVA) followed by the Tukey post hoc test were used to analyze statistical significance ($p < 0.05$).

The concentration of the extract that inhibits 50% of the enzyme activity (IC_{50}) was calculated. Extracts with high inhibitory activity were analyzed using a series of suitable extract concentrations. IC_{50} values were determined by plotting percent inhibition (Y axis) versus log10 extract concentration (X axis) and calculated by logarithmic regression analysis from the mean inhibitory values. Regression analysis by a dose response curve was plotted to determine the IC_{50} values.

RESULTS

In this study, the inhibitory effects of extracts against nitric oxide (-NO) radical were investigated. Tables 2-4 were shown the antioxidant activity for NO radical of three EM groups. It was observed that inhibition percentage values go on increasing with enhancements in concentration of research EM and collagen extracts in the assay mixture. The NO activity of the EM-A was the highest among tested EM in the manufactures. NO scavenging activity of the EM-A was evaluated 60.0% on 100% solution without collagen and that of the EM-B was 47.6% at same concentration. NO scavenging activity of the EM-C was evaluated 44.7% at same concentration. Although EM-A was slightly higher in NO inhibitory activity than those of EM-B and EM-C, there was no significant difference among three groups ($p > 0.05$) (Table 5). Three EM groups with collagen were shown higher radical scavenging effects at all concentration than those of EM groups without collagen. There was significant difference between EM groups with collagen and EM groups without collagen ($p < 0.01$).

Figure 1 was shown the rate of NO inhibitory of Quercetin (positive control) and relative inhibitory rate for three EM groups on 100% EM. The values for the EM-A, EM-B, and EM-C were 87.2%, 68.2%, and 66.2%, respectively. IC_{50} value was inversely related to the antioxidant activity of EM and collagen extracts. The values of IC_{50} for the EM-A, EM-B, and EM-C were 264.6, 303.5, and 358.1 µg/ml, respectively (Table 6).

Overall, EM-A (*Lactobacillus plantarum* as lactic acid bacteria) and EM-B (: *Lactobacillus casei* as lactic acid bacteria) was slightly higher in NO inhibitory activity

than those of EM-C (*Streptococcus lactis* as lactic acid bacteria). However, there was no significant difference among three groups ($p > 0.05$).

Table 2: The degree of inhibition (%) of NO by EM-A with collagen at different concentrations.

Concentration of EM (%)	Collagen (mg)				
	0	25	50	75	100
6.25	9.34±1.87	18.90±2.14	30.69±0.55	39.71±3.89	44.68±3.15
12.5	22.12±3.15	29.35±1.76	39.30±3.34	49.30±2.69	50.59±3.67
25.0	36.32±3.75	45.85±3.08	49.87±4.02	55.10±3.54	61.45±4.51
50.0	46.77±3.72	52.99±1.27	56.84±2.94	60.24±2.54	65.58±3.24
75.0	56.37±3.86	58.82±2.55	60.28±1.71	63.77±2.38	69.46±2.77
100.0	59.96±1.90	62.90±0.80	63.49±1.11	66.23±1.85	72.43±2.04
F-test	7.781**				

Data represented the mean ± SD from three replicates. **, $p<0.01$.

Table 3: The degree of inhibition (%) of NO by EM-B with collagen at different concentrations.

Concentration of EM (%)	Collagen (mg)				
	0	25	50	75	100
6.25	7.77±1.31	15.71±1.15	28.15±2.93	34.94±2.83	39.20±2.23
12.5	18.78±3.42	27.45±2.44	39.70±2.84	43.61±2.05	47.71±3.88
25.0	24.66±1.75	36.93±3.01	47.07±3.99	49.13±1.31	54.41±2.99
50.0	33.79±0.87	42.36±2.78	53.75±3.85	56.98±2.45	59.12±3.11
75.0	42.16±1.61	49.08±0.88	57.76±3.32	61.27±2.56	64.23±1.04
100.0	47.55±2.97	53.46±1.24	61.22±3.12	64.06±1.83	68.20±1.93
F-test	8.242**				

Data represented the mean ± SD from three replicates. **, $p<0.01$.

Table 4: The degree of inhibition (%) of NO by EM-C with collagen at different concentrations.

Concentration of EM (%)	Collagen (mg)				
	0	25	50	75	100
6.25	5.46±1.69	15.04±2.66	28.61±0.72	33.70±1.45	38.38±0.89
12.5	13.31±3.84	23.95±2.36	37.29±0.91	41.75±1.92	45.87±2.07
25.0	18.61±4.96	34.03±1.44	45.98±0.90	48.98±1.85	53.43±2.58
50.0	27.20±3.50	40.09±1.80	53.95±1.01	54.84±1.76	57.86±2.80
75.0	39.20±2.79	44.70±0.53	57.79±0.81	59.57±0.82	63.38±2.55
100.0	44.46±2.91	47.87±1.38	61.36±0.98	62.72±1.07	66.17±2.75
F-test	8.696**				

Data represented the mean ± SD from three replicates. **, $p<0.01$.

Table 5: Compare *F*-test of difference in means of three samples by EM and collagens at same concentration.

Collagen (mg)	EM + collagen		
	A	B	C
0	0.082	0.024	0.053
25	0.002	0.011	0.039
50	0.084	0.255	0.005
75	0.251	0.118	0.051
100	0.308	0.133	0.138

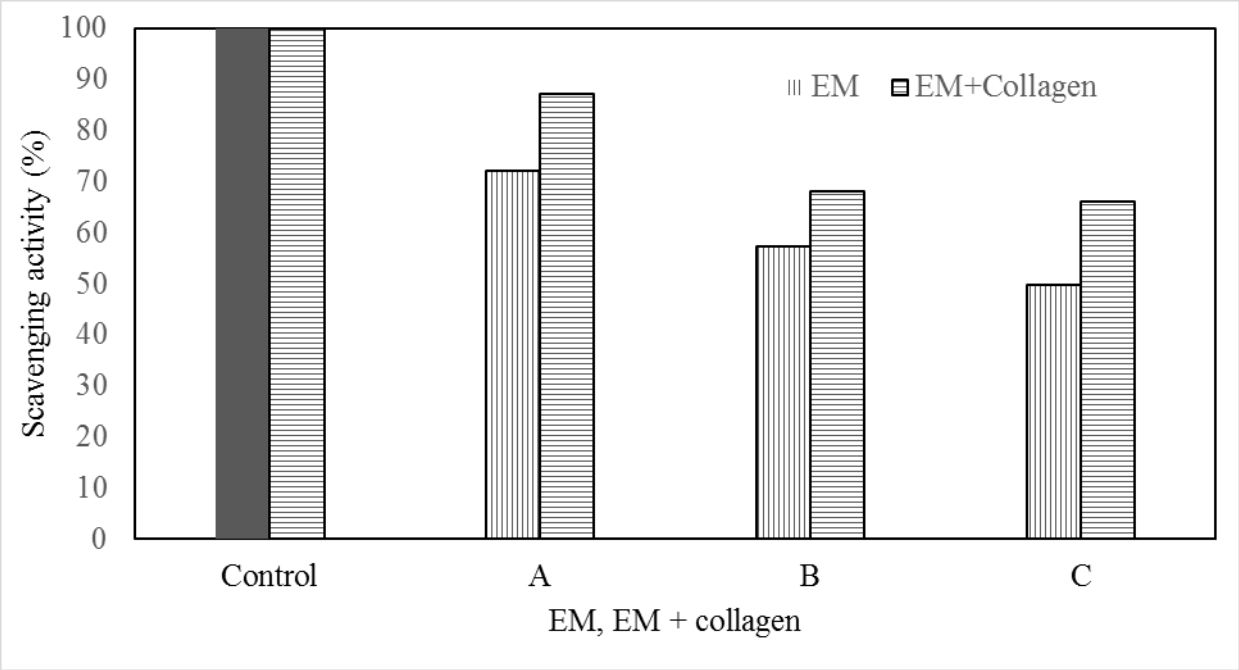

Figure 1: The rate of NO inhibitory of Quercetin (positive control) and relative inhibitory rate on 100% EM.

Table 6: The 50% inhibition (IC_{50}) of NO on EM and collagen.

EM	Without Collagen	With Collagen
A	264.6	254.2
B	303.5	284.3
C	358.1	320.8

DISCUSSION

Urmi and Sariah (2006) studied that there are numerous benefits to using effective microorganisms in agriculture.[13] Effective microorganisms contribute to soil enrichment, by harmonizing and diversifying native microorganisms.[14]

The collagen produced by organisms is defined as endogenous, which consists of three long helicoidally shaped chains of amino acids.[15] Collagen has a unique triple-helix structure with a repeated amino acid sequence of (Gly-X-Y)n, in which X and Y are typically Pro and Hyp.[16] It can be found in various food sources added as a denatured form, gelatin, or, alternatively, manufactured as dietary supplements in an enzymatically hydrolyzed form, collagen hydrolysate. Collagen can be largely divided into meat extract and fish extract. Collagen extracted from fish has a smaller molecular weight than collagen extracted from meat and has a 42 times higher absorption rate, according to the Japan Cell Improvement Medicine Association. Collagen can't be absorbed by the body in its whole form. This means collagen proteins must be broken down into smaller peptides or amino acids before they can be absorbed. Collagen supplements are typically already broken down into peptides of two or three amino acids for the purpose of increasing their bioavailability. Low-molecular-weight peptides could be applied into food systems, for example, beverages with hydrolyzed collagen show a great advantage of easy digestion, high assimilation (about 80%), and good absorption at the intestinal level.[17]

Peptides, produced by collagen hydrolysis, act as antifreeze, are reported to have superior inhibitory effects against ice recrystallization, and are known to have antioxidant effects.[18-19] In addition, the collagen types obtained from various sources have been confirmed to have a similar cryo-protective effect by protecting the cell membrane.[20] Collagen derived from fish scales with potential effects was employed, and low and high molecular weight collagens were compared to determine suitable conditions for various lactic acid bacteria.[21] Protein digestion is a very efficient process that depends on the nature of the protein and the proteins or peptides that undergo catalysis by bacterial proteases and peptidases to release shorter peptides and amino acids.[22]

Mammalian collagenases separate the alpha-chain at specific and limited sites (i.e., Gly-Ile) to denature the molecule, and nonspecific proteolytic enzymes take over and digest sever the collagen fragment.[23-24] Bacterial collagenases sever the alpha-chain at several sites.

CONCLUSIONS

Overall, EM-A (*Lactobacillus plantarum* as lactic acid bacteria) and EM-B (: *Lactobacillus casei* as lactic acid bacteria) was slightly higher in NO inhibitory activity than those of EM-C (*Streptococcus lactis* as lactic acid bacteria). Conclusion, it is estimated that microorganisms in the EM fermentation liquid decomposed collagen to enhance NO antioxidant function.

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