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Protective effect of CSN1S2 protein of goat milk on ileum microstructure and inflammation in rat-CFA-induced rheumatoid arthritis

Rista Nikmatu Rohmah[1], Edi Widjajanto[2], Fatchiyah Fatchiyah[1*]

[1]*Department of Biology, Faculty of Science, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia*

[2]*Department of Biomedical, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia*

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ABSTRACT

Objective: To observe the protective effect of goat milk alpha (S2)-casein (CSN1S2) protein on ileum microstructure and inflammation in rat-complete Freund's adjuvant-induced rheumatoid arthritis model.

Methods: Twenty four male Wistar rats were divided into six groups of two models. The body weight, food intake and albumin level of all subjects were calculated. The ileum microstructures were analyzed by scanning electron microscopy. Histopathological analysis was observed by hematoxylin-eosin staining and the level expressions of immunoglobulin E, secretory immunoglobulin A, interleukin-17, interleukin-10, Ki-67 and caspase-9 were measured by using western blotting.

Results: CSN1S2 protein of milk or yogurt could repair the ileum villi of rat arthritis group similar to the normal. The level expressions showed the immunoglobulin E, secretory immunoglobulin A, interleukin-17 and caspase-9 decreased in milk CSN1S2 protein and yogurt CSN1S2 protein rat groups. The level expression of interleukin-10 was increased, and also Ki-67 was significantly increased in milk CSN1S2 protein and yogurt CSN1S2 protein rat groups. CSN1S2 protein of milk and yogurt could increase the body weight and albumin significantly, meanwhile food intake increased but not significantly.

Conclusions: CSN1S2 protein of goat milk and yogurt could repair the ileum microstructure, suppress inflammatory process and also increase the body weight, food intake and albumin level. This result indicates that goat CSN1S2 protein may protect the ileum disorder in rheumatoid arthritis disease.

1. Introduction

The understanding of rheumatoid arthritis (RA) diseases recently has been characterized by inflammation at joint synovial[1]. RA is a systemic inflammatory disease that causes an increased risk of developing co-morbidity conditions[2]. Several studies on RA patients showed declining of body cell mass with increasing of body fat mass, prior to malnutrition characteristic[3]. Those conditions are caused by high levels of inflammatory substances, which are produced at inflammatory site[4,5].

In addition, the previous study reported that there was gut inflammation on RA patients. The increasing self-reactive antibodies and pro-inflammatory T-lymphocytes were considered to contribute to the gut inflammation[6]. Furthermore, the imbalance of gut micro-biota could influence T cell responses and caused systemic inflammation on RA patients[6,7]. However, the interaction between RA disease and ileum inflammation is still unclear and needs further research.

Functional food derived from bioactive peptides is one of the healthy and beneficial food sources, such as bovine or goat milk, and various meat, fish or plants. Those foods have provided neonates and adults with important physiological mechanism[8]. This peptide was suggested as immunomodulatory and anti-inflammatory agents[9]. *In-vitro* studies on culture of mammalian cells showed the effect of bioactive peptides that could induce cell growth[10]. Study by animal inflammatory model demonstrated that milk peptide could decrease the intestinal inflammation[11]. Bovine and goat milk contains a lot of bioactive peptides from proteins of α-casein-S1, α-casein-S2 (CSN1S2), β-casein and κ-casein. Our previous study found that Ethawah breed goat milk and yogurt contain a bioactive peptide with protein profile of 36 kDa which was suspected as the CSN1S2 protein[12]. Though, the effect of goat milk CSN1S2 protein function on RA is not clear

*Corresponding author: Fatchiyah Fatchiyah, Department of Biology, Faculty of Science, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia.
Tel: 62341 575841
E-mail: fatchiya@ub.ac.id, fatchiya@gmail.com
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yet.

The Ethawah breed goat CSN1S2 protein might repair the ileum disorder and influence cytokine expression on the inflammatory mechanism of RA. This study focused on exploration of the effect of bioactive peptide CSN1S2 in RA rats fed by the Ethawah breed goat milk and yogurt on the ileum performance and inflammatory mechanism.

2. Material and methods

2.1. Isolation of CSN1S2

Milk and yogurt were taken from Ethawah breed goat milk, at UPTD Indonesian local goat, and Singosari, Malang. Isolation of milk and yogurt goat CSN1S2 protein was performed according to the previous study[13] with some modifications. The protein was measured by using Nano drop spectrophotometer.

2.2. Experimental animals

Twenty four male Wistar rats were divided into six groups of two models. The RA rats were given trough subcutaneous injection (SIGMA, USA) of 100 μL complete Freund's adjuvant (CFA). Fourteen days later, the treatment was continued with intradermal injection of 50 μL CFA (SIGMA, USA). The two rat models, normal and RA rat models had three groups in each. In normal rat model, there were Group N, Group NM (normal rats induced with milk CSN1S2 protein) and Group NY (normal rats treated with yogurt CSN1S2 protein), while where were Group RA, Group RAM (RA rats treated with milk CSN1S2 protein) and Group RAY (RA rats treated with yogurt CSN1S2 protein) in RA rat model. Then the rats were treated with specific CSN1S2 protein of goat milk and yogurt that were given orally every morning for 3 months with a dose of 2 mg/kg body weight with some modification according previous study[14]. After 3 months, rats ileum's were taken and kept on 4% paraformaldehyde. All animal conditions and handling were approved by Ethical Clearance KEP-90-UB.

2.3. Microstructure analysis

The ileums were proceeded for microstructure observation by using scanning electron microscopy (SEM) based on Mahdy *et al.*[15] with some modifications. The samples were observed by using SEM (Hitachi, TM300, Tokyo) with 2 000× magnification.

2.4. Histopathological analysis

Hematoxylin-eosin staining was conducted for histopathological analysis based on previous method[16] with some modifications. The staining results were observed by using microscope inverted BX53 (Olympus) with 600× magnification.

2.5. Body weight and food intake analysis

Body weight of all subjects was calculated as average of body weight measurement during 3 months. Besides that, the food intake of all subjects was also measured in relation with body weight. Furthermore, food intake was measured as average of food weight differences before and after given to the subjects. This calculation was also conducted during 3 months.

2.6. Albumin level

The serum albumin level was determined based on Hayashi *et al.*[3] with some modification.

2.7. Western blotting

Sample proteins of ileum from all groups were isolated by using procedure that already described before[17] with some modifications and then separated by using 15% of sodium dodecyl sulfate polyacrylamide gelelectrophoresis. To identify the cytokine expression of ileum from all rat groups, western blotting analysis was conducted with specific primer antibodies, and several antibodies used were mouse-anti-immunoglobulin E (IgE) (1:1 500, antibodies-online.com), mouse-anti-secretory immunoglobulin A (SIgA), mouse-anti-interleukin (IL)-10 (1:1 500, Bioss, Inc.), rat-anti-IL-17, mouse-anti-Ki67, and mouse-anti-caspase-9 (1:1 500, Santa Cruz Biotechnology, Inc). The protein band from sodium dodecyl sulfate-polyacrylamide gelelectrophoresis gel was transferred to polyvinylidene fluoride membrane, then the membrane was washed by using phosphate buffered saline tween-20 and incubated by using secondary antibody-labeled-AP (1:2 500 on thermomorphic biphasic amine solvent, Gathering MD, USA) for 1 h at room temperature. After that membrane was exposed prior to target protein which was labeled by using western-blue-substrate solution of nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate (Gathering MD, USA), the density of specific protein was measured by Bio-Rad Quantity One software.

2.8. Statistical analysis

The density of protein expressions was analyzed by using SPSS 16.0 Base system for windows (SPSS, Inc., Chicago). The values were presented as mean ± SD. The statistical analysis was performed by using One-way ANOVA test. In this study, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Microstructure of ileum microvilli

SEM examination of ileum mucosal microstructures from normal group showed that the surface was intact with regular shape of microvilli (Figure 1a).

In contrast, the RA group showed the characteristic of smooth surface, without any microvilli, decrease in surface density and expansion of the cavities (Figure 1d). In general, the normal rats treated with the CSN1S2 proteins from Ethawah breed goat milk showed similar surface to the normal ileum (Figure 1b). Different result was shown at the ileum surface of the normal group after treatment of CSN1S2 proteins from Ethawah breed goat yogurt, whereas the ileum surface exhibited a little cavity structure that was identical to villi-shape performance of ileum microstructure in RA group (Figure 1c). The RA rats treated with CSN1S2 proteins from Ethawah breed goat milk had the ability to recover the cavity that appeared, meanwhile the microstructure of RA rats treated by the CSN1S2 proteins from Ethawah breed goat milk had low ability to reform the cavity shape (Figures 1e and 1f). The data were supported by histological ileum that showed the villi destruction on columnar absorption cell and provided the repairing of villi ileum similar to

the normal appearance after treatment of CSN1S2 milk or yogurt although yogurt presented a low ability than CSN1S2 milk (Figure 2).

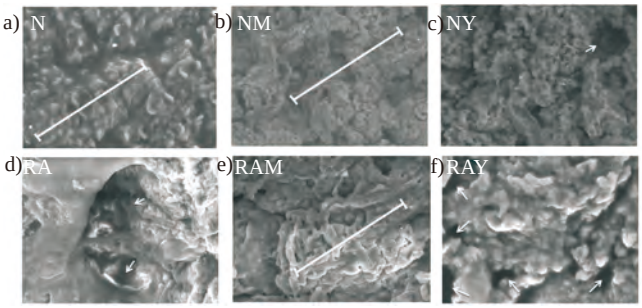

Figure 1. Microstructure of rats' ileum villi in six groups exposed by SEM.

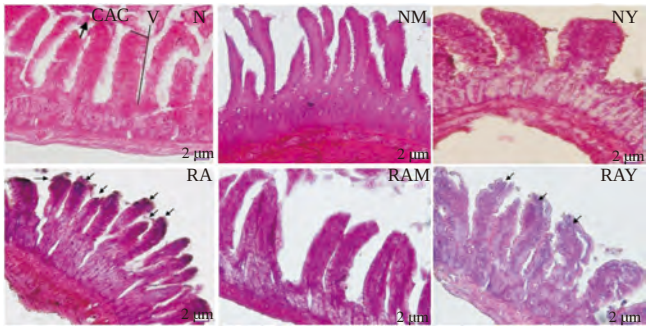

Figure 2. Histopathology of rats' ileum villi in normal and RA rat groups visualized by BX53 Microscope.

3.2. Immunoglobulin and cytokine expression levels

Effect of CSN1S2 protein was observed through the expression levels of immunoglobulins and cytokines. Although the expression levels of IgE [(0.56 ± 0.04) INT/mm^2] and sIgA in RA group [(0.62 ± 0.03) INT/mm^2] were higher than other groups, statistically there was no significant differences among them ($P > 0.05$). The IgE and sIgA expression levels in RA group were down-regulated after rats were induced by both CSN1S2 milk and yogurt proteins of Ethawah breed goat: IgE of RAM was (0.47 ± 0.03) INT/mm^2, IgE of RAY was (0.52 ± 0.03) INT/mm^2, sIgA of RAM was (0.32 ± 0.05) INT/mm^2, and sIgA of RAY was (0.33 ± 0.04) INT /mm^2. These results were close to the levels of IgE (0.37 ± 0.02) INT/mm^2 and sIgA (0.40 ± 0.04) INT/mm^2 in normal group (Figure 3b).

As shown in Figure 3c, several cytokines of inflammation represented by Il-17 and IL-10 were investigated by using western blotting method. Normal group [IL-17 was (0.310 ± 0.002) INT/mm^2, IL-10 was (0.160 ± 0.007) INT/mm^2] showed reducing level of IL-17 (0.27 ± 0.02) INT/mm^2 and elevating level of the IL-10 (0.32 ± 0.05) INT/mm^2 after treated with CSN1S2 milk protein of Ethawah breed goat. Meanwhile, the IL-17 level in RA group treated with CSN1S2 yogurt protein was (0.47 ± 0.04) INT/mm^2 and IL-10 in RA group [(0.110 ± 0.009) INT/mm^2] was slightly lower than normal. The expression level of IL-10 was slightly up-regulated [RAM was (0.13 ± 0.01) INT/mm^2 and RAY was (0.19 ± 0.03) INT/mm^2] and IL-17 expression was down-regulated [RAM was (0.37 ± 0.03) INT/mm^2 and RAY was (0.26 ± 0.02) INT/mm^2] in both of CSN1S2 proteins from Ethawah breed goat milk and yogurt in RA rat groups.

The levels of Ki-67 and caspase-9 in RA rat groups were also in contrast with the expression in normal rat groups after CSN1S2 protein treatments (Figure 3d). The increasing of Ki-67 expression level [NM was (0.140 ± 0.003) INT/mm^2 and NY was (0.140 ± 0.003) INT/mm^2] as proliferation marker was slightly elevated and balanced by caspase-9 level as early cell-death marker [NM was (0.085 ± 0.003) INT/mm^2 and NY was (0.072 ± 0.002) INT/mm^2]. Interestingly, in RA rat groups, the expression of Ki67 was increased ($P < 0.05$) by CSN1S2 milk protein [RAM was (0.100 ± 0.005) INT/mm^2] and CSN1S2 yogurt protein [RAY was 0.050 ± 0.001 INT/mm^2] than RA. The caspase-9 expression in RA rats was decreased significantly [RA became (0.0700 ± 0.0005) INT/mm^2 in RAM and (0.1000 ± 0.0008) INT/mm^2 from (0.140 ± 0.003) INT/mm^2 ($P < 0.05$) in RA](Figure 3d).

3.3. Effect of inflammation on several biological characteristics

The mean body weight in RA rats (307.78 ± 35.76) kg was reduced

Table 1

Body weight, food intake, and sera albumin test for the different group normal, RA, and treated- CSN1S2.

Parameters	N	NM	NY	RA	RAM	RAY
Body weight (kg)	371.45 ± 40.61^a	353.94 ± 29.94^b	337.93 ± 31.21^c	307.78 ± 35.76^d	316.00 ± 36.40^e	315.20 ± 24.10^d
Food intake (g/rat/day)	23.07 ± 11.16	21.89 ± 11.83	22.29 ± 13.44	21.35 ± 11.11	21.81 ± 12.21	20.73 ± 12.76
Albumin (g/L)	3.55 ± 0.04^b	3.72 ± 0.02^a	3.64 ± 0.01^{ab}	3.35 ± 0.12^c	3.54 ± 0.05^b	3.38 ± 0.08^c

Values are presented as mean ± SD. All groups were analyzed using ANOVA followed by Tukey test with significant different $P < 0.05$. $P < 0.05$ in comparison with normal group; $P < 0.05$ in comparison with NM group; $P < 0.05$ in comparison with NY group; $P < 0.05$ in comparison with RA group; $P < 0.05$ in comparison with RAM group; $P < 0.05$ in comparison with RAY group.

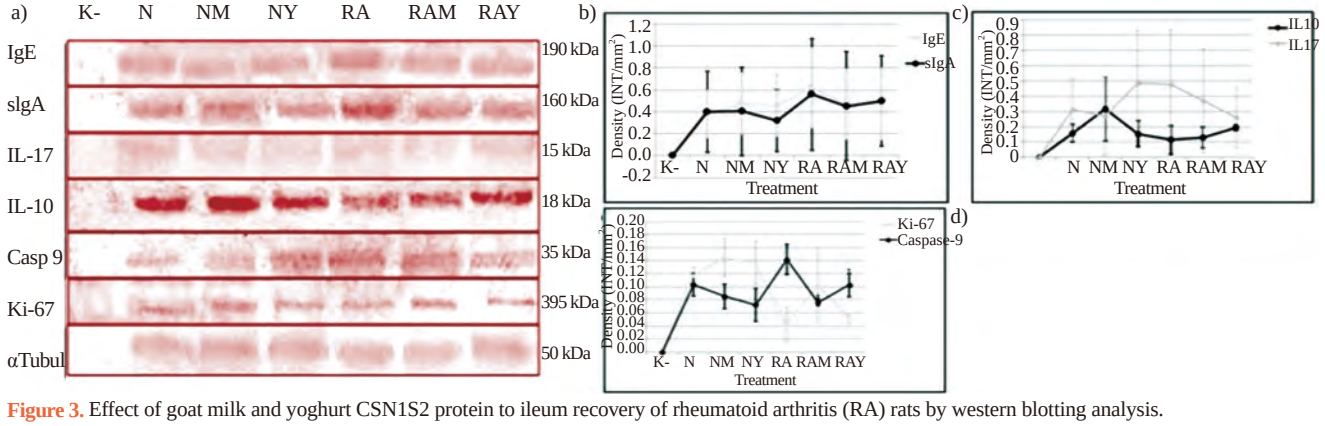

Figure 3. Effect of goat milk and yoghurt CSN1S2 protein to ileum recovery of rheumatoid arthritis (RA) rats by western blotting analysis.

a: Expression levels of IgE, sIgA, IL-17, IL-10, Ki-67 and caspase-9 of ileum, α-tubulin as positive control; b: Density of IgE and sIgA; c: Density of IL-17 and IL-10; d: Density of Ki-67 and caspase-9; K-: Negative control.

significantly compared with normal group (371.45 ± 40.61) kg (Table 1). The rats in group RA treated with the CSN1S2 proteins from Ethawah breed goat milk and yogurt showed increasing body weight which was close to normal [RAM = (316.00 ± 36.40) kg, RAY = (315.20 ± 24.10) kg]. In contrast, the food intake levels in all treatment groups were not significant different compared to control group. The albumin level was decreased significantly in RA rats (3.35 ± 0.12) g/L compared with normal (3.55 ± 0.04) g/L. The albumin level in RA rats treated with CSN1S2 milk and yogurt was increased significantly similar to normal [RAM = (3.54 ± 0.05) and RAY = (3.38 ± 0.08) g/L].

4. Discussion

The study revealed that there were different microstructures of ileum characteristic between normal and RA rat models. The RA rat model was showed incompact and smooth surface of ileum structure compared with the normal rats. The ileum histology also has shown the villi ileum destruction on columnar absorption cell. Mostly, the RA patient had gastrointestinal disorder caused by imbalance in the gut micro-biota. This condition related with adjuvant could change T-cell responses and stimulate systemic inflammation[6,7]. The adjuvant could increase gut permeability that facilitated luminal antigen flow through gut mucosal immune system[18]. The RA model had high caspase-9 and low Ki-67 compared with normal. This result indicated that the ileum destruction may also be caused by unbalanced intestinal homeostasis through the different levels between apoptosis and proliferation. Pathogenesis in RA was characterized with increasing of apoptosis and inflammatory mechanism on synovial membrane[19]. Whereas, in this study we found the same mechanism in ileum.

CSN1S2 goat milk treatment group showed a repairing of villous ileum with intact surface appearance which was similar to normal but CSN1S2 yogurt treatment still had some cavities. Our result elucidates that the peptides of CSN1S2 protein of Ethawah breed goat milk have biological function as immunomodulatory, but the response is different from CSN1S2 yogurt protein. We suggested that the peptides of CSN1S2 yogurt protein of Ethawah breed goat had different mechanism to control physiological function that probably failed to control flora imbalance of small intestine[20]. This study supported that pro-biotic was not appropriate for routine use for patients with inflammatory bowel disease[21].

Proliferation and apoptosis were related with cell recovery and rejuvenation[19,22]. CSN1S2 milk and yogurt proteins revealed their role on RA cell recovery. Both treatments were successful to elevate cell rejuvenation with increasing level of proliferation and significantly decreasing apoptosis level. This result suggested that the milk and yogurt CSN1S2 protein could control homeostasis through regulating the cellular apoptosis and proliferation on rat ileum RA.

Immunoglobulin is one factor associated with RA onset in patients. The previous study reported the high level of IgE on sera of RA patients[23]. The IgE level on mast cells induced rapidly the release of the inflammatory mediator, such as histamines, cytokines, proteases, chemotactic factors, and also some metabolites[24] since tissue destruction and injury will be contributed to joint inflammation. The level of IgE in ileum RA model in this study also showed high level and we suggested that it also affected the ileum inflammation. Our study showed the elevating of IgE level in normal model and decreasing in RA model treated with CSN1S2 milk and yogurt proteins. The increasing of IgE level in normal model may indicate the response to CSN1S2, without any inflammation as the same as previous study[25] that goat milk still was possible to induce an allergy with low percentage. The decreasing IgE level in RA group fixed the CSN1S2 goat milk which presents the potency as anti-allergy to control the homeostasis.

Secretory-IgA level in serum of RA patients was increasing as a response to CFA which stimulated innate and adaptive immunity against mycobacterial antigens[26]. Innate immunity was exposed by sIgA level on mucosal defense. The function of sIgA level in RA model is as anti-inflammatory agent to control intestinal homeostasis[27].

The ileum destruction was also related with alteration of inflammatory cytokines. The increasing of IL-17 level and decreasing of IL-10 in RA model explained that there was inflammation response. This process was related with the role of CFA as inflammatory agent that could lead an adaptive immunity which stimulated T helper cells to release IL-17 and induced inflammatory response[28]. IL-17 and IL-10 have antagonist expression in inflammatory event. CSN1S2 milk and yogurt proteins could suppress inflammatory effect indirectly through increasing of IL-10 against IL-17. Recent study also reported that IL-10 could suppress the Th-17 in turn and decrease the IL-17 level[29]. Vice versa, treatment with CSN1S2 yogurt in normal control could induce the inflammatory effect by increasing IL-17 level. This evidence supported that yogurt did not have stable result both in ileum microstructure and IL-17 expression.

In this study, we observed several markers related with malnutrition, such as, body weight loss, food intake and albumin level in serum. Interesting results were shown on the food intake and weight loss measurements. Although food intake measurement results from all treatments were not significantly different, from body weight loss measurement, it was significantly different in each treatment. Body weight losses are correlated with food intake[30]. It is suggested that other pathways were related with the body weight losses such as the inflammation[3]. The inflammatory factors may promote the dysfunction gastrointestinal and associated with malabsorption alteration. Therefore, it suggested that those mechanisms in turn could decrease the body weight[31]. Our result also showed the decrease of serum albumin significantly. The current study demonstrated that the malnourished children had low albumin[32,33]. Previous study showed that decreased albumin level may be correlated with alteration of disease activity on RA condition related with high level of pro-inflammatory cytokines[3]. CSN1S2 protein of Ethawah breed goat milk and yogurt could influence body weight and the increase serum albumin level. This result indicated that the CSN1S2 protein could influence catabolic effect that was related with inflammation process through body weight, food intake and increasing albumin.

In conclusion, based on our results, the bioactive peptide of CSN1S2 protein of Ethawah breed goat milk and yogurt could repair the ileum destruction and also suppress the inflammatory process on ileum through induction as anti-inflammatory agent. We suggest that the goat milk CSN1S2 protein could improve the repairing process more properly than protein from yogurt. Further research related with safe dose consumption of milk, especially yogurt, is still needed.

Conflict of interest statement

We declare that we have no conflict of interest.

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