Effects of a Casein Hydrolysate Prepared from *Aspergillus oryzae* Protease on Adjuvant Arthritis in Rats

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We evaluated the effects of a casein hydrolysate (CH) prepared from *Aspergillus oryzae* protease on rat adjuvant arthritis, a model of human rheumatoid arthritis. CH was administered orally once a day to the animals for 22 d after the adjuvant injection. CH suppressed swelling in the adjuvant-unjected hind paws, and a higher dose of CH suppressed the increase in arthritic score and swelling of the adjuvant-injected hind paws. A histopathological examination revealed evidence that the higher dose of CH suppressed the articular changes in the rats. In addition, CH suppressed the production of nitric oxide and prostaglandin E2 in the plasma of the rats. These results suggest that CH had a suppressive effect on adjuvant arthritis by inhibiting the acute and chronic inflammatory reactions.

Key words: casein hydrolysate; *Aspergillus oryzae* protease; adjuvant arthritis; nitric oxide; prostaglandin E2

Human rheumatoid arthritis is a chronic inflammatory disease which is characterized by synovial cell proliferation and massive infiltration of leukocytes.1,2 Such immunological abnormalities as the dysfunction of suppressor T cells are considered to be involved in the disease.3 Non-steroidal anti-inflammatory drugs (NSAIDs) are used for treating rheumatoid arthritis in spite of their gastric and renal toxicity.4 Although NSAIDs have beneficial effects on pain and edema, they have no effect on the basic process in the joint lesions.5 On the other hand, disease-modifying anti-rheumatic drugs (DMARDs) have been reported to have therapeutic effects on rheumatoid arthritis by improving the immunological abnormalities.6,7 However, the therapeutic effects of DMARDs are also not always satisfactory, because of their efficacy and side effects.

In the previous study, the authors had reported that some food components such as glucosamine8 and α-linked galactooligosaccharide9 were effective on the joint lesions in rat adjuvant arthritis, a model of human rheumatoid arthritis. Hayashida et al.10 have recently demonstrated that lactoferrin, a ubiquitous protein present in milk, was also effective for treating the animals. It was explained that an oral administration of this substance had efficacy on the arthritis mediated by the anti-inflammatory and/or immunomodulatory action.8–10 The results disclosed evidence that the substance may have efficacy as a functional food on rheumatoid arthritis in humans.8–10

Various physiological functional peptides (bioactive peptides) such as opioid peptides,11 antibacterial peptides,12 immunostimulating peptides,12 antioxidative peptides,13 and angiotensin-I converting enzyme (ACE) inhibitory peptides,14,15 have been reported from a protein hydrolysate, synthetic peptides, and fermented products. In the present study, we prepared a casein hydrolysate (CH) from casein prepared with the proteolytic enzyme from *Aspergillus oryzae* (*A. oryzae*) which contained a large amount of oligopeptides with 2 to 3 amino acid residues and free amino acids.16 It has been demonstrated that such peptides as l-valyl-l-prolyl-l-proline (VPP) and l-isoleucyl-l-prolyl-l-proline (IPP) had potent antihypertensive activities by their ACE-inhibitory effects.17,18 These active peptides would not usually be produced by digestion with endogenous gastrointestinal enzymes, when casein is taken as a food.16 Therefore, novel physiological functions would be observed for the administration of peptides prepared by digestion with microbial enzymes.

Based on these findings, we hypothesized that CH might have a therapeutic effect on inflammatory disorders in the joints. In the present study, we orally
administered CH to adjuvant arthritic rats, and evaluated the effects on the progression of inflammation. The results disclose evidence that CH suppressed the progression of arthritis in rats and that CH inhibited the production of nitric oxide (NO) and prostaglandin E$_2$ (PGE$_2$).

Materials and Methods

Animals. Female Lewis rats were purchased from Charles River Laboratories Japan (Atsugi, Japan) and housed for 7 d. The animals were kept in the SPF condition (room temperature, 24 ± 3°C; relative humidity, 55 ± 15%; 12 h light/12 h dark illumination cycle). The rats were fed with a standard feed (MF, Oriental Yeast, Tokyo, Japan) with free access to water at all times. Eight-week-old rats were randomly allocated to 4 to 6 groups and given the treatment. All procedures using the animals were in accordance with the “Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science,” and were approved by the Animal Use and Care Committee at Mercian Cleantec Corporation.

Preparation of the test substances. CH was prepared by Calpis Co., Ltd., using A. oryzae protease as described previously. Sodium caseinate (CNA) prepared from cow’s milk was dissolved in hot water (80°C) to an 8% (wt/vol) solution, and the pH value was adjusted to 7.0 by adding 1N NaOH. After cooling to <20°C, the protease isolated from A. oryzae was added directly to the CNA solution at a final concentration of 0.32% (wt/vol), and the mixture incubated at 50°C for 14 h. The reaction was stopped by heating the solution at 100°C for 5 min to inactivate the enzyme, and the hydrolysate was powdered with a spray drier before use. CNA and a mixture of free amino acids were respectively purchased from Arla Foods Ingredients (Viby J, Denmark) and Wako Pure Chemical Industries (Osaka, Japan) for use as negative control substances. The mixture of free amino acids was based on the amino acid composition of casein (CAA, as shown in Table 1). Casein was hydrolyzed for 24 h in evacuated sealed tubes with constant-boiling HCl containing 0.1% phenol at 105°C, and then the hydrolysate was analyzed with an amino acid analyzer (Shimadzu LC-20A, Kyoto, Japan). Asp and Gln of CAA included the hydrolysates of Asn and Gln. Ibuprofen (Wako Pure Chemical Industries), an NSAID, was employed as a positive control drug. Each of these substances was dissolved or suspended in a 0.5% carboxymethylcellulose solution (CMC, Wako Pure Chemical Industries).

Induction of arthritis and administration of the test substance. Adjuvant arthritis was induced by an intra-articular injection of 0.5 mg of heat-killed Mycobacterium tuberculosis H37Ra cells (Difco Labs., Detroit, MI, USA) that had been emulsified with liquid paraffin (Wako Pure Chemical Industries). The emulsion was injected into the footpad of the right hind paw of each rat on day 0. In the first experiment, the effect of CH (1,000 mg/kg BW) was compared with those of CNA and CAA (each 1,000 mg/kg BW). These substances were administered orally once a day to 4 groups of 8 rats for 22 d after injecting the adjuvant. In the second round of the experiment, in order to examine the dose-dependent effect of CH, CH (500, 1,000 and 2,000 mg/kg BW) or ibuprofen (30 mg/kg BW) was administered to 6 groups in the same manner. The control rats received the vehicle alone in each experiment. The normal (not inducted) rats did not receive the adjuvant injection in the dose-response experiment.

Clinical evaluation of arthritis. The rats were clinically observed for their characteristic signs and symptoms. The rats were scored by grading each paw, except the adjuvant-injected paw (right hind), from 0 to 4 based on the erythema, swelling and rigidity of the joint (0 = nil; 1 = mild; 2 = moderate; 3 = moderately severe; 4 = severe). The maximum possible score was 12 (4 points for each paw, except the right hind). The volume of swelling was also measured in both hind paws with a foot volume meter (TK-105, Muromachi Kikai Co., Ltd., Tokyo, Japan).

Histopathological evaluation of arthritis and the plasma analysis. At the end of the dose-response experiment (day 22), the rats were anesthetized with diethyl ether. Blood samples were taken from each rat by cardiac puncture with sodium heparin. The blood was centrifuged at 2,200 × g for 10 min at 4°C, and then the supernatant was stored at −80°C as blood plasma.

The limbs were then dissected and fixed in 10% neutral buffered formalin. After decalcification with saturated ethylene diamine tetracetic acid (EDTA), the

<table>
<thead>
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<th>Amino acid</th>
<th>% (w/w)</th>
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<tr>
<td>Ile</td>
<td>5.22</td>
</tr>
<tr>
<td>Leu</td>
<td>8.94</td>
</tr>
<tr>
<td>Lys</td>
<td>7.56</td>
</tr>
<tr>
<td>Met</td>
<td>2.77</td>
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<tr>
<td>Cys</td>
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</tr>
<tr>
<td>Phe</td>
<td>4.79</td>
</tr>
<tr>
<td>Tyr</td>
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<tr>
<td>Thr</td>
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<td>Pro</td>
<td>10.65</td>
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<tr>
<td>Ser</td>
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*Asp and Glu in CAA included the hydrolysates of Asn and Gln.

Table 1. Amino Acid Composition of Casein Analyzed by an Amino Acid Analyzer
joints were longitudinally sectioned, and tissue sections (10 μm) were mounted on glass slides and stained with hematoxylin and eosin. Articular lesions were observed under an optical microscope. Histopathological findings being graded into four levels (− = intact, + = mild, ++ = moderate, +++ = severe). Multiplication of the synovial lining cells was evaluated as "intact," three layers or less; "mild," four or five layers; "moderate," six to eight layers; and "severe," nine layers or more of the cells.10

Plasma samples were used for measuring the NO and PGE2 levels. Total NO (nitrite and nitrate) was measured by the Griess method, using a nitrite/nitrate CII colorimetric assay kit (Dojin Chemical Labs., Kumamoto, Japan), and PGE2 was measured by an enzyme immunoassay (EIA), using a prostaglandin monoclonal E2 EIA kit (Cayman Chemical Company, MI, USA).

Statistical analysis. The result of each experiment is expressed as the mean ± standard error (mean ± SE). Fisher’s PLSD test or Kruskal-Wallis test followed by the Mann-Whitney test were used to establish the significance between the experimental groups, as appropriate. A p value of less than 0.05 was considered to be statistically significant.

Results

Body weight change with adjuvant arthritis in the rats

In the first experiment, the animals lost body weight slightly in all the groups treated with adjuvant. No significant differences were apparent in the body weight between the vehicle control and groups treated with CH, CNa and CAA throughout the experimental period (Fig. 1A).

In the dose-response experiment, all the adjuvant-treated groups lost body weight compared to the normal group which had not been injected with the adjuvant (p < 0.001). No significant differences were apparent between the vehicle control and groups treated with CH and ibuprofen, with the exception being on day 14 (1,000 mg/kg BW of CH) (Fig. 1B).

Arthritic score and foot pad volume with adjuvant arthritis in the rats

In the vehicle control group, the adjuvant-uninjected paw joints started to show erythema, swelling and rigidity on day 9, the arthritic score reaching the maximum level on day 19 or day 21 in both experiments. CH (1,000 mg/kg BW) suppressed the arthritic score on days 14, 19 and 21 in the first experiment (p < 0.05 compared with the vehicle control, Fig. 2A). The same dose of CNA and CAA (1,000 mg/kg BW) had no effect on the arthritic score in the first experiment. CH (1,000 mg/kg BW) suppressed the arthritic score in the first experiment, and the same dose of CH slightly suppressed it in the dose-response experiment, although not significantly (Fig. 2B). The discrepancy seemed to depend on the maximum score levels in the vehicle control groups between the first experiment (10.4 ± 0.4) and dose-response experiment (11.5 ± 0.4). In the dose-response experiment, a higher dose of CH (2,000 mg/kg BW) significantly suppressed the arthritic score on days 9, 14, 19 and 21 (p < 0.05 or 0.01 compared with...
the vehicle control). The suppressive effect of CH (2,000 mg/kg BW) was almost the same as that of ibuprofen (30 mg/kg BW). These findings suggest that CH and ibuprofen both had a beneficial effect on the clinical signs and symptoms of adjuvant arthritis, and that CNa and CAA had no effect on the animals.

In the dose-response experiment, the volume of swelling was measured in the bilateral hind paws to examine the precise effect of CH on the animals. The lower dose of CH (500 mg/kg BW) significantly suppressed swelling in the adjuvant-injected right hind paw on days 9, 19 and 21, and the higher dose of CH (2,000 mg/kg BW) suppressed it from day 5 to day 21, with the exception of day 14 (Fig. 3A). On the other hand, CH
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(500, 1,000 and 2,000 mg/kg BW) suppressed swelling in the adjuvant-uninjected left hind paw in a dose-dependent manner. Notably, CH (1,000 and 2,000 mg/kg BW) suppressed swelling in the uninjected left hind paw from day 9 to day 21 (p < 0.05 or 0.001 compared with the vehicle control, Fig. 3B). Ibuprofen (30 mg/kg BW) suppressed swelling in both the adjuvant-injected right and -uninjected left hind paws from day 9 to day 21 (p < 0.001 compared with the vehicle control, Fig. 3B). Thus, CH and ibuprofen could both suppress the inflammatory reaction in adjuvant arthritis.

**Table 2.** Histopathological Evaluation of Adjuvant Arthritic Rats Treated with the Casein Hydrolysate (CH) or Ibuprofen

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CH 2,000 mg/kg BW</th>
<th>Ibuprofen 30 mg/kg BW</th>
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<tbody>
<tr>
<td>Multiplication of</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>synovial lining cell</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Subsynovial soft tissue edema</td>
<td>0</td>
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<td>8</td>
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<tr>
<td>Fibrin exudation</td>
<td>0</td>
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<td>Fibroblast proliferation</td>
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<tr>
<td>Lymphocyte infiltration</td>
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<td>2</td>
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<tr>
<td>Cartilage degeneration</td>
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<td>4</td>
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<tr>
<td>Bone/cartilage replaced</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pannus formation</td>
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<td>8</td>
<td>0</td>
</tr>
<tr>
<td>New bone formation</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Periostitis</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

See the legend to Fig. 4. The histopathological evaluation was carried out on the adjuvant-injected right hind paw. The histopathological findings were graded into four levels (−, intact; +, mild; ++, moderate; ++++, severe). *Number of rats.
In the time-course study, adjuvant arthritis had two phases: i) local inflammation (acute phase or first inflammation) reached a maximum level 4–5 days after adjuvant injection and was characterized by swelling of the injected paw, and ii) systemic change (chronic phase or second inflammation) was apparent 7–14 d after the injection, and chronic lesions expanded to the other paws.20,21) In addition, it is well known that NSAIDs suppress the acute and chronic phases in inflammation,22) while DMARDs selectively suppress the chronic phase.

**Fig. 4.** Histopathological Changes in Adjuvant Arthritic Rats Treated with the Casein Hydrolysate (CH) or Ibuprofen.

At day 22, the limbs were dissected from each rat and fixed in 10% neutral buffered formalin. After decalcification with saturated EDTA, the joints were sectioned longitudinally, and tissue sections were mounted on glass slides and stained with hematoxylin and eosin. Articular lesions were observed under an optical microscope. A. In the adjuvant-injected hind paw, the non-treated control rats demonstrated severe articular changes. Multiplication of the synovial lining cells (M), subsynovial soft tissue edema (E) and pannus formation (P) are indicated by arrows (×4). B. In the adjuvant-injected hind paw, the articular changes were almost normal in the adjuvant arthritic rats who had received treatment with 2,000 mg/kg BW of CH (×4). C. In the adjuvant-injected hind paw, articular changes were almost normal in the adjuvant arthritic rats who had received treatment with 30 mg/kg BW of ibuprofen (×4).

In the time-course study, adjuvant arthritis had two phases: i) local inflammation (acute phase or first inflammation) reached a maximum level 4–5 days after adjuvant injection and was characterized by swelling of the injected paw, and ii) systemic change (chronic phase or second inflammation) was apparent 7–14 d after the injection, and chronic lesions expanded to the other paws.20,21) In addition, it is well known that NSAIDs suppress the acute and chronic phases in inflammation,22) while DMARDs selectively suppress the chronic

**Fig. 5.** Nitric Oxide (NO) and Prostaglandin E₂ (PGE₂) Production in Adjuvant Arthritic Rats Treated with the Casein Hydrolysate (CH) or Ibuprofen.

At the end of the experiment (day 22), blood samples were taken from each rat through cardiac puncture with sodium heparin. The blood was centrifuged, and then the supernatant was stored as blood plasma. Total NO (nitrite and nitrate) was measured by the Griess method with a CII colorimetric nitrite/nitrate assay kit, and PGE₂ was measured by an enzyme immunoassay (EIA), using a prostaglandin monoclonal E₂ EIA kit. A. NO level in plasma obtained from adjuvant arthritic rats on day 22 after the adjuvant injection. B. PGE₂ level in plasma obtained from adjuvant arthritic rats on day 22 after the adjuvant injection. Data represent the mean from 8 animals, and vertical bars represent S.E. "p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle control."
In the present study, CH (500, 1,000 and 2,000 mg/kg BW) had efficacy during the chronic phase (from day 9 to day 21) in the adjuvant-uninjected paw in a dose-dependent manner (Fig. 3B), while the higher dose (2,000 mg/kg BW) of CH suppressed both the acute and chronic phase reactions (from day 5 to day 21) in the adjuvant-injected paw (Fig. 3A). CH had different effects on the adjuvant-injected paw and -uninjected paw, which seems to have depended on the administered dose. The histopathological examination of the joints disclosed evidence that CH suppressed both acute and chronic inflammation such as lymphocyte infiltration, subsynovial soft tissue edema, fibroblast proliferation and bone/cartilage being replaced by connective tissue (Fig. 4). Moreover, the suppressive effect of CH in the acute phase of inflammation was consistent with our preliminary experimental data that the oral administration of CH suppressed the carrageenan-induced edema, a model of acute inflammation in rats. In the present study, ibuprofen (30 mg/kg BW) suppressed the acute phase reaction in the adjuvant-injected paw and that of chronic phase lesions in the adjuvant-injected and -uninjected paws. Thus, the effect that of CH in alleviating the progression of adjuvant arthritis was different from those of ibuprofen. These results suggest that CH had a therapeutic effect on adjuvant arthritis by an anti-inflammatory and/or immunomodulating action.

It is known that NO produced by cartilage and synovial cells is implicated in the pathogenesis of human osteoarthritis and rheumatoid arthritis. NO is a potent chemical mediator that plays an important role in immunity and inflammation. NO has been reported to play an important role in the pathogenesis of arthritis in animal models, including adjuvant arthritis in rats, and an inhibitor of NO synthase has been reported to have a suppressive effect on arthritis in experimental animal models. On the other hand, PGE2 is also one of the important inflammatory mediators in rheumatoid arthritis. When tissues are exposed to diverse physiological and pathological stimuli, arachidonic acid is liberated from the membrane phospholipids by phospholipase A2 and converted to prostaglandin H2 (PGH2) by prostaglandin H synthase (cyclooxygenase). PGH2 is converted to major prostanoids including PGE2 by enzymes. It has been well explained that NSAIDs had inhibitory actions on cyclooxygenase (COX) which has been found in two isoforms (COX1 and COX2). In the present study, CH (500, 1,000 and 2,000 mg/kg BW) reduced the plasma NO level in the animals in a dose-dependent manner (Fig. 5A). The higher dose of CH (2,000 mg/kg BW) significantly reduced the plasma PGE2 level (Fig. 5B). Ibuprofen (30 mg/kg BW) also reduced the plasma NO and PGE2 levels. Therefore, the reduction in plasma levels of NO and PGE2 by CH may be associated with an attenuation of the inflammatory reaction in the rats. We examined the effects of CH on the lipopolysacharide-induced production of interleukin-1β, interleukin-6 and tumor necrosis factor-α in macrophages and splenocytes from the adjuvant arthritic rats in this study. However, there was no significant difference, suggesting that CH had no effect on the production of cytokines (data not shown).

It has been shown that β-casein (1–28) purified from a commercial casein phosphopeptide preparation enhanced the proliferative response of human T, B, and monocyte cell lines, and stimulated IgA production of the B cell line. Twenty food-protein hydrolysates (a containing casein hydrolysate) hydrolyzed with gastrointestinal proteinases have been demonstrated to exert antimicrobial and immunostimulatory activities. In addition, a tryptic casein hydrolysate had an immunostimulatory action by enhancing the phagocytosis of mouse peritoneal macrophages and blood phagocytic cells. On the other hand, as CH exhibited an anti-inflammatory and/or immunosuppressive effect, the biological activity of CH was different from these protein hydrolysates with an immunostimulatory action. Furthermore, these β-casein (1–28) or casein hydrolysates prepared with gastrointestinal proteases, which were almost completely composed of endoproteases, would have consisted of larger peptides than that with A. oryzae protease which mainly contained a large amount of oligopeptides with 2 to 3 amino acid residues and free amino acids. In the present study, CH might have included novel short peptides which were able to suppress inflammation and/or immunostimulation.

In conclusion, we found that CH suppressed the progression of adjuvant arthritis by inhibiting acute and chronic inflammation and the production of inflammatory mediators (NO and PGE2). Thus, CH can be expected as a novel and safe substance for treating human rheumatoid arthritis. Although the precise mechanism of action remains unclear, we believe that these results might be helpful to understand the mode of action of CH and to encourage further related studies. Further experiments will be required to purify and identify the active substance(s) in the CH mixture.

References


33) Spangler, R. S., Cyclooxygenase 1 and 2 in rheumatic

