Influence of the treatment used in inflammatory bowel disease on the level of protease activity

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Abstract

Introduction: There is growing evidence that intestinal proteases have a role in the pathogenesis of gastrointestinal inflammatory diseases. IBD, which includes Crohn's disease (CD) and ulcerative colitis (UC), has an additional source of proteases represented by infiltrated and activated inflammatory cells. The aim of our study was to determine proteolytic system activity in patients with CD and UC. We limited the number of proteases tested by determining proteases active in acidic, neutral and alkaline pH.

Material and methods: The study included 40 patients with IBD – 20 CD patients and 20 UC patients. Among the 20 CD patients, 17 were treated with aminosalicylates, 14 with azathioprine, and 4 with corticosteroids, while 8 patients were undergoing biological treatment. Among the 20 UC patients, 19 were treated with aminosalicylates, 8 with azathioprine, and 3 with corticosteroids. The optimal pH in which the enzymes were active was determined in acidic, neutral, and alkaline buffer environments. We prepared buffers of defined pH from 2.2 to 12.8, separated by 0.2 intervals and then determined proteolytic activity against substrates (gelatine, haemoglobin, ovalbumin, albumin, cytochrome C, and casein).

Results: A decrease was observed in the activity of acid proteases (pH 5), alkaline proteases (pH 7), and neutral proteases (pH 7.6 and 8.6) in the groups of CD patients in remission in comparison with the active phase.

In the group of patients with CD treated biologically, acid protease activity (pH 5.0) was lower than in CD patients not receiving biological treatment.

Activity of neutral (pH 7.0) and alkaline (pH 7.6 and 8.6) proteases in the plasma of patients with UC in remission were lower in comparison to the active phase.

Activity of acid (pH 5.0) and alkaline (8.6) protease inhibitors was higher in CD patients in the active phase in comparison to remission. In UC patients with exacerbation of the disease, the activity of alkaline (pH 8.6) protease inhibitors was increased compared to remission.

Conclusions:

1. Our research may suggest that the immunomodulatory treatment used in IBD, aimed at reducing the level of leukocytes, may contribute to a reduction in protease activity.

2. The decrease in the level of proteases in patients with CD and UC in remission may be a marker suggesting the patients' response to the treatment.

Introduction:

Inflammatory bowel disease (IBD), which includes mainly ulcerative colitis (UC) and Crohn's disease, is a chronic and recurrent inflammatory condition of the gastrointestinal tract [1].

Various factors have been associated with the development of IBD. Polymorphisms in a number of genes have been shown to impair functions such as lymphocyte activation, autophagy, pathogen sensing, stress response, antigen presentation and chemotaxis. The presence of these genetic variants may contribute to an imbalance in the immune response and increased predisposition to IBD [2].

Protease genes constitute about 2% of the mammalian genome, and their gene products are enzymes considered essential for processes such as development, coagulation, immunity, inflammation and cell death [3].

The four categories of proteinases, cysteine, serine, aspartic, and metalloproteinases, are named and classified according to their essential catalytic component, usually an amino acid, in their active site [4].

Proteases are important mediators in gastrointestinal physiology, produced and released by the pancreas to be activated in the intestinal lumen for digestive purposes. Proteolytic activity is also found in mucosal tissues in healthy conditions, where it plays a role in mucus consistency and mucosal antigen processing [5].

Bacteria, helminths and yeasts potentially present in the intestinal lumen also produce and release proteases. Mast cells release various forms of proteases: tryptase, chymase, cathepsin G and granzyme B. Resident macrophages also produce and release various forms of proteases: matrix metalloproteinases (MMPs), caspase, and cathepsins D and L. In the inflamed gut, inflammatory cells are another major source of proteases, which they use to degrade extracellular tissues and intracellular particles, increasing their phagocytic properties. For example, neutrophils release massive amounts of elastase, proteinase-3 and cathepsin G5. Additionally, all resident cells of the GI tract express intracellular proteases, i.e. caspases, which have fundamental roles in cell apoptosis, and proteolytic enzymes – autophagins, responsible for autophagy processes [6].

Proteases are subject to strict regulation. Most proteases are synthesized as inactive zymogens that become activated through a number of mechanisms, including cleavage by upstream proteases, dimerization or changes in pH. Proteases are also subject to inactivation by endogenous inhibitors, and thus the balance of active and inactive proteases is tightly controlled [7].

In intestinal pathophysiological contexts such as inflammatory bowel disease, proteolytic homeostasis can be disrupted in tissues [5]. There is growing evidence that intestinal proteases have a role in the pathogenesis of gastrointestinal inflammatory disease [3].

IBDs, which include CD and UC, have an additional source of proteases represented by infiltrated and activated inflammatory cells. Some studies have shown that expression of a very large number of proteases is upregulated in IBD [6]. The expression and activity of certain MMPs is increased during acute inflammation, but an imbalance between MMPs and their natural tissue inhibitors (TIMPs) has also been reported for IBD [8]. Protein or mRNA expression of proteases from infiltrated immune cells (neutrophil elastase, tryptase, proteinase-3, cathepsin G, chymase or granzymes) has also been shown to increase in

inflamed tissues from patients with IBD. Inappropriate induction of cell death through apoptosis or autophagy is also associated with IBD, and proteases involved in such processes (caspases and autophagins) are upregulated in IBD, particularly UC [6].

Given the large number of proteases that have been identified and their diverse functions in the development of IBD, it is quite difficult to specify which proteases are responsible for the manifestation of the pathological condition [6]. The aim of our study was to determine proteolytic system activity in patients with CD and UC. We limited the number of proteases tested by determining proteases active in acidic, neutral and alkaline pH.

Material And Methods:

The study included 40 IBD patients diagnosed in the Gastroenterology Ward and Gastroenterology Clinic of the Cardinal Stefan Wyszyński Regional Specialist Hospital in Lublin. The control group consisted of 20 healthy subjects (9 men and 11 women, 19–62 years of age). The IBD group consisted of 20 CD patients (11 men and 9 women, aged 19–52), and 20 UC patients (12 men and 8 women, 17–46).

Among the 20 CD patients, 17 were treated with aminosalicylates, 14 with azathioprine, and 4 with corticosteroids, while 8 patients were undergoing biological treatment (6 patients with Humira and 2 patients with Inflectra). The duration of the disease ranged from 0.5 to 16 years. There were 12 patients currently suffering exacerbation of the disease.

Among the 20 UC patients, 19 were treated with aminosalicylates, 8 with azathioprine, and 3 with corticosteroids. The duration of the disease ranged from 0.5 to 11 years. There were 11 patients currently experiencing exacerbation.The material for the study consisted of plasma isolated from peripheral blood collected from the 40 IBD patients and the 20 healthy individuals.

The total protein concentration was assayed by the Lowry method, as modified by Schacterle and Pollack [9]. The optimal pH for activity of the enzymes was assayed in acidic, neutral, and alkaline buffer environments. We prepared buffers of defined pH, from 2.2 to 12.8, separated by 0.2 intervals, as discribed by Łoś and Strachecka [10]. Then proteolytic activity against substrates (gelatine, haemoglobin, ovalbumin, albumin, cytochrome C, and casein) was tested by the Anson [11] method as modified by Grzywnowicz et al. [12] and Strachecka et al. [13,14]. The results indicated that haemoglobin was the optimal substrate for further analyses. Activity of acid, neutral and alkaline proteases in their optimal pH were determined according to the method described by Anson [11], as modified by Strachecka et al. [14]. Following the addition of diagnostic inhibitors, i.e. pepstatin A, PMSF, iodoacetamide, and o-phenantroline, proteolytic activity was determined according to Lee and Lin [15]. Natural inhibitors of acid, neutral and alkaline proteases were determined according to Lee and Lin [15]. The detailed protocols are described by Łoś and Strachecka [10].

The data were analysed using the Statistica 13.3 statistics package. The significance level was set at 5% ($p \leq 0.05$). The Mann-Whitney U test and Kruskal-Wallis test H were carried out.

Results:

Acid proteases exhibited optimal activity at pH 5, neutral proteases were active at pH 7, and alkaline proteases were active at pH 7.6 and 8.6 (Fig. 2). Asparagine, serine and thiol proteases as well as metalloproteases were present in the plasma of subjects in all groups. The protease inhibitors were active only at pH 5 and 8.6.

Discussion:

Excessive or poorly controlled inflammation is the central aspect of IBD. Host factors which regulate this inflammatory response play a crucial role in the pathophysiology of IBD [16]. The activity of proteases must be strictly regulated to prevent inappropriate and often destructive proteolysis. Excessive and uncontrolled protease activity by endogenous inhibitors can lead to many disease states [17]. Proteases associated with IBD exert proinflammatory effects: they potentiate the proinflammatory properties of cytokines and chemokines and remodel the extracellular matrix to enable leucocyte infiltration. Proteases degrade tight junction proteins, inducing plasma extravasation and increased intestinal permeability, and induce apoptosis in intestinal epithelial cells [6]. Dysregulated proteolytic activity leads to homeostatic imbalance in the body. For this reason a number of strategies have been developed to control proteolysis, including spatial and temporal regulation, zymogen activation, protease degradation, and inhibition of proteases by macromolecular inhibitors [17]. Our research showed that plasma protease activity differed in patients with CD and UC as compared to the controls. Activity of neutral (pH 7) and alkaline (pH 7.6) proteases was higher in the group of CD patients than in the controls, while the activity of acid (pH 5) and alkaline (pH 8.6) proteases was lower. These differences in protease activity may be due to the effects of the treatment used in the group of patients. In the group of patients with UC, the reduction in alkaline protease activity (pH 8.6) was statistically significant compared to the control.

Several studies indicate an increase in proteases in inflammatory bowel disease [16]. The statistically lower activity of proteases in our patients with IBD in comparison to the control group may be a response to treatment. Drugs used to treat IBD have been shown to have the capacity to induce apoptosis in T cells or monocytes in vitro and in vivo [18]. Consequently, treatment may have reduced the level of proteases in the study group of patients.

Evidence has shown that T helper type 1 (Th1)-associated cytokines such as interferon (IFN)-γ and TNF-α, as well as Th17-associated cytokines such as interleukin (IL)-17A and IL-23, play an important role in the pathogenesis of IBD. The disequilibrium between pro- and anti-inflammatory cytokines and increased infiltration of activated immune cells such as T cells, B cells, natural killer cells, macrophages or neutrophils in the intestinal mucosa further aggravate mucosal inflammation in IBD [19]. For this reason, we divided the patients in our study according to whether their disease was active or in remission, and the results were quite interesting. We observed a decrease in acid protease (pH 5), alkaline protease (pH 7) and neutral protease (pH 7.6 and 8.6) activity in the group of CD patients in remission in comparison with the active phase (Fig. 4). Activity of neutral (pH 7.0) and alkaline (pH 7.6 and 8.6) proteases in the

plasma of patients with ulcerative colitis in remission were also decreased in comparison to the active phase (Fig. 7). Our research may suggest that the treatment used in IBD is to a large degree directed at reducing activated proteases.

Sulfasalazine is drug considered a mainstay therapy for Crohn's disease or UC. It is composed of 5-ASA and a sulfapyridine moiety. Recent work indicates that sulfasalazine is a biologically active substance with potent anti-inflammatory effects on intestinal epithelial cells or T lymphocytes. Sulfasalazine has been shown to interfere with regulation of apoptosis by inhibiting the NFκB inflammatory pathway. In an experimental study, Doering et al [20] showed that sulfasalazine had a potent proapoptotic effect on T lymphocytes in CD patients [20]. This treatment, with potent anti-inflammatory effects and a proapoptotic effect on intestinal T cells, was used in our group of patients with IBD. In addition, azathioprine was used to treat the CD and UC patients.

The most commonly used agents in the management of IBD are azathioprine (AZA) and mercaptopurine (MP). AZA and MP induce apoptosis in activated lymphocytes [21]. The thiopurine drugs, 6-mercaptopurine (6-MP) and its prodrug azathioprine (AZA) remain the mainstay of immunomodulatory therapy for IBD and are indicated in steroid-dependent and refractory patients as prophylaxis in CD. Their use is often limited, because 30% to 50% of patients discontinue them due to side effects or lack of clinical efficacy. The lack of response to these immunomodulators has been attributed to differences in individual variations in drug metabolism [22].

In the group of patients with CD treated biologically, acid protease activity (pH 5.0) was lower than in Crohn's patients not receiving biological treatment (Fig. 6).

The lower protease activity in patients with CD treated biologically, using Inflectra (infliximab) and Humira (adalimumab), may be the result of treatment directed at proinflammatory TNF-α, which additionally contributed to the reduction in CD protease levels. A recent study has shown that increased matrix metalloproteinase activity in tissues from IBD patients was restored to control levels after infliximab treatment [6]. Researchers have induced production of matrix metalloproteinase-9 (MMP-9) by culturing cells at acidic pH (5.4-6.5) (Kato et al. 2005). [23]

This is consistent with our observations, in which activity of acid proteases (pH 5) was significantly increased in the group of patients with active CD (Fig. 4) and in CD patients not receiving biological treatment (Fig. 6), which may indicate increased MMP-9 activity in the patients. Increased MMP-9 activity may result in significant degradation of type IV collagen, the main component of the vascular basement membrane, a physical barrier preventing cells from migrating, thereby enabling leukocyte migration during inflammation (Lipka, Boratyński 2008).[24]. Biological therapy currently involves monoclonal antibodies directed against specific targets implicated in the pathogenesis of chronic inflammatory conditions. For IBD, this primarily encompasses approved anti-tumour necrosis factor (TNF) therapies (adalimumab, infliximab, golimumab or certolizumab), as well as agents, approved or under development, that target integrins (vedolizumab and natalizumab) or interleukin (IL)-12/23 (ustekinumab) [25]. The lack of significant differences in the activity of acid proteases (pH 5) in the UC groups in the active phase

of the disease and in remission (Fig.7) may be due to differences in the two diseases. Despite some overlapping clinical features, these diseases are defined by separate inflammatory profiles and symptomatology and differ in gut microbiota composition [26]. Microorganisms produce a vast array of aspartic, cysteine, metallo-, and serine proteases. However, it is not clear which commensal bacteria can secrete proteases that damage the mucosal barrier [27]. Intestinal antimicrobial/microbial composition can influence treatment outcomes [28]. Treatment choices for UC or CD also differ because they are entities with different pathophysiological aspects [29]. One drug used to treat CD, but not UC, is methotrexate, which inhibits folic acid metabolism, required for many cell functions, including purine synthesis, and acts as a powerful inhibitor of cell metabolism and mitosis [28][30]. CD and UC also differ in their response to biological treatment. In CD, adalimumab is effective in the clinical setting and in patients with an attenuated response to infliximab, but it is less efficacious in UC [31].

Our analyses showed significantly lower activity of protease inhibitors in CD and UC patients in remission (Fig. 5 and 8). This may be linked to the treatment used or decreased efficacy or expression of endogenous protease inhibitors, which can lead to upregulation of protease expression and the development of inflammation. Recent work has focused on the association of endogenous protease inhibitors with IBD pathology [32]. During the disease process the balance between proteases and their inhibitors is shifted, leading to altered spatial and temporal control of substrate cleavage [33].

Intestinal tissues from CD and UC patients showed elevated proteolytic activity. This could be due either to upregulated protease expression or to decreased efficacy or expression of endogenous proteases inhibitors [32]. Our research may suggest that the immunomodulatory treatment used in IBD may affect the activity of proteases as well as their inhibitors by reducing inflammation and leukocyte levels.

One feature of the inflamed mucosa in IBD is the formation of cryptitis and abscesses, resulting from an influx of neutrophils into the epithelial area and intestinal lumen, and thus the accumulation of neutrophils, which is closely correlated with the activity of the disease [19].

Our study showed that protease activity in CD and UC patients was significantly lower in the remission stage, which involves a reduction in the inflammatory state and a reduced influx of neutrophils into the epithelial area. This may suggest that the reduction in proteases in the CD and UC group may also be associated with the reduction in inflammatory markers in response to treatment.

Conclusions:

1. Our research may suggest that the immunomodulatory treatment used in IBD, aimed at reducing leukocyte levels, may contribute to a reduction in protease activity.

2. The decrease in the level of proteases in patients with CD and UC in remission may be a marker of the patients' response to the treatment.

Declarations:

Ethical approval and consent to participate

All patients were recruited following written informed consent, the protocol was approved by the Local Bioethics Committee in Lublin KE-0254/179/2016.

Consent for publication

Not applicable.

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None.

Authors' contributions

ED designed the study and collected data. AS, MG performed the laboratory work. JB performed the statistical analysis. ED, AS analyzed the data and drafted the manuscript. PGK, JK, NS edited and revised the manuscript.

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used/or analyzed during the current study are included in this published article.

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Figures

Protein concentrations in the plasma of patients with CD were significantly increased in comparison to the control group of healthy subjects and the UC patients (Fig. 1).

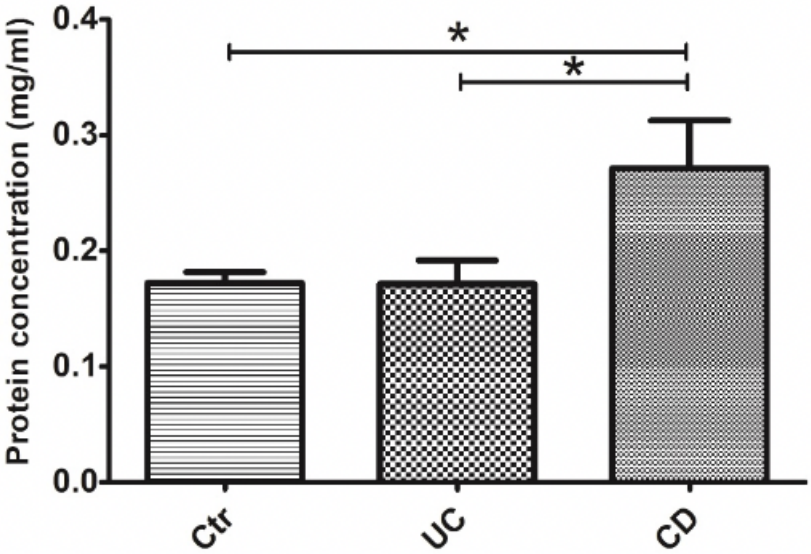

Figure 1

Protein concentration in the plasma of patients with Crohn's disease and ulcerative colitis (±SD). The differences are statistically significant for comparisons between groups (control, CD and UC) at $P \leq 0.05$.

Activity of acid proteases (pH 5.0) was decreased in CD patients in comparison to the healthy group. Alkaline proteases (pH 8.6) were decreased in the plasma of CD and UC patients in comparison to the control group. Neutral (pH 7) and alkaline (pH 7.6) proteases in CD patients were increased in comparison to the control group (Fig. 2).

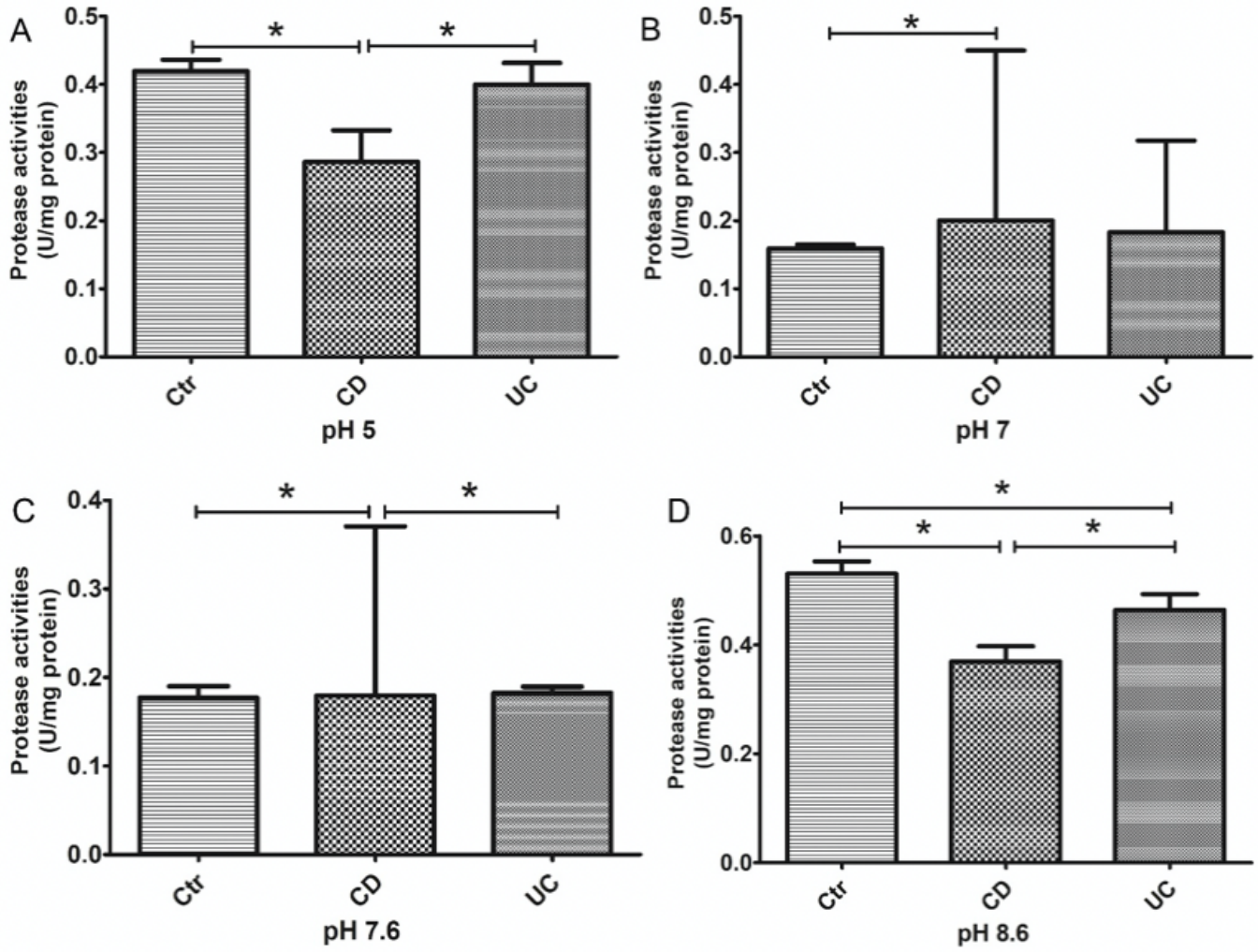

Figure 2

Activity of acid A (pH 5.0), neutral B (pH 7.0) and alkaline C and D (pH 7.6 and 8.6) proteases in the plasma of patients with Crohn's disease (CD) and ulcerative colitis (UC) (±SD). The differences are statistically significant for comparisons between groups (control, CD and UC) at $P \leq 0.05$.

The activity of acid (pH 5.0) and alkaline (8.6) protease inhibitors in the plasma of patients with Crohn's disease and ulcerative colitis showed a statistically significant decrease in comparison to the healthy group. The lowest inhibitory activity was observed in the group of patients with CD (Fig. 3).

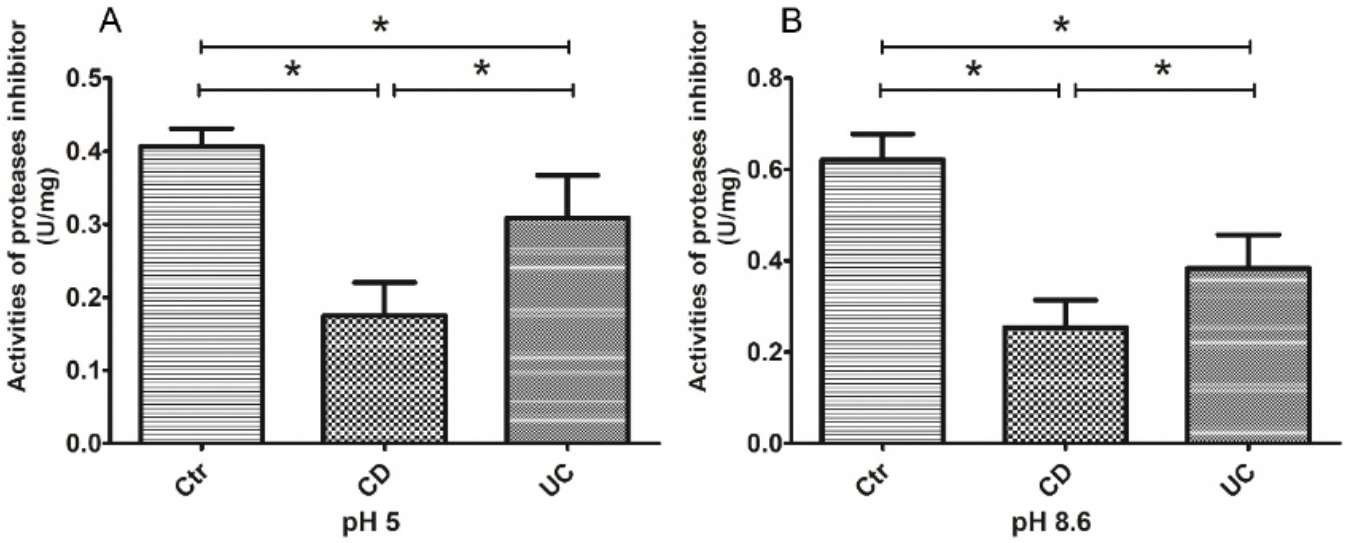

Figure 3

Activity of acid A (pH 5.0) and alkaline B (8.6) protease inhibitors in the plasma of patients with Crohn's disease (CD) and ulcerative colitis (UC) (±SD).

A decrease in acid protease (pH 5), alkaline protease (pH 7) and neutral protease (pH 7.6 and 8.6) activity was observed in the groups of CD patients in remission in comparison with the active phase (Fig. 4).

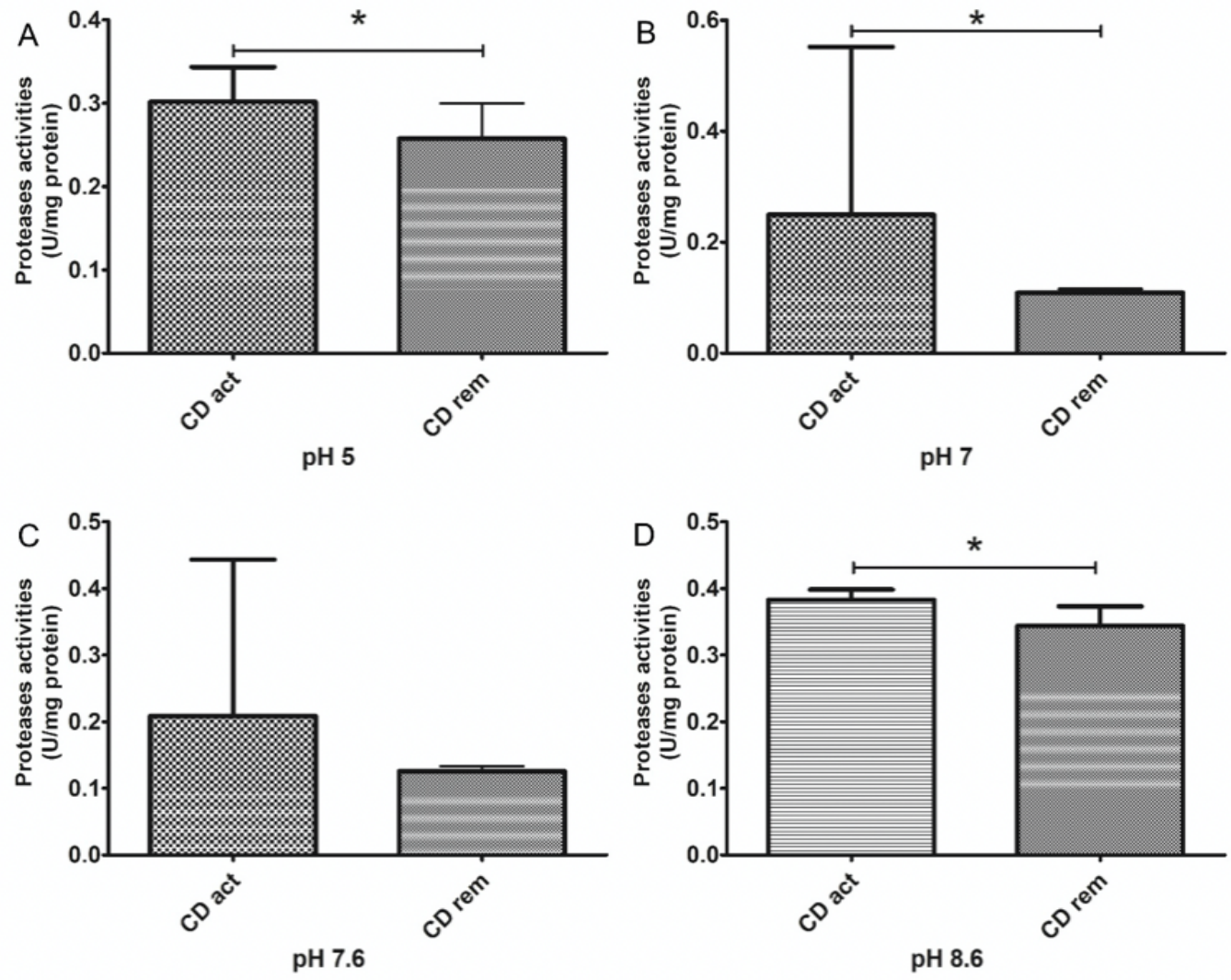

Figure 4

Activity of acid A (pH 5.0), neutral B (pH 7.0) and alkaline C and D (pH 7.6 and 8.6) proteases in the plasma of patients with Crohn's disease – active (act) and in remission (rem) (±SD). The differences are statistically significant for comparisons between groups (act and rem) at $P \leq 0.05$.

Activity of acid A (pH 5.0) and alkaline B (8.6) protease inhibitors in patients with the active phase of CD is increased in comparison to patients in remission (Fig. 5).

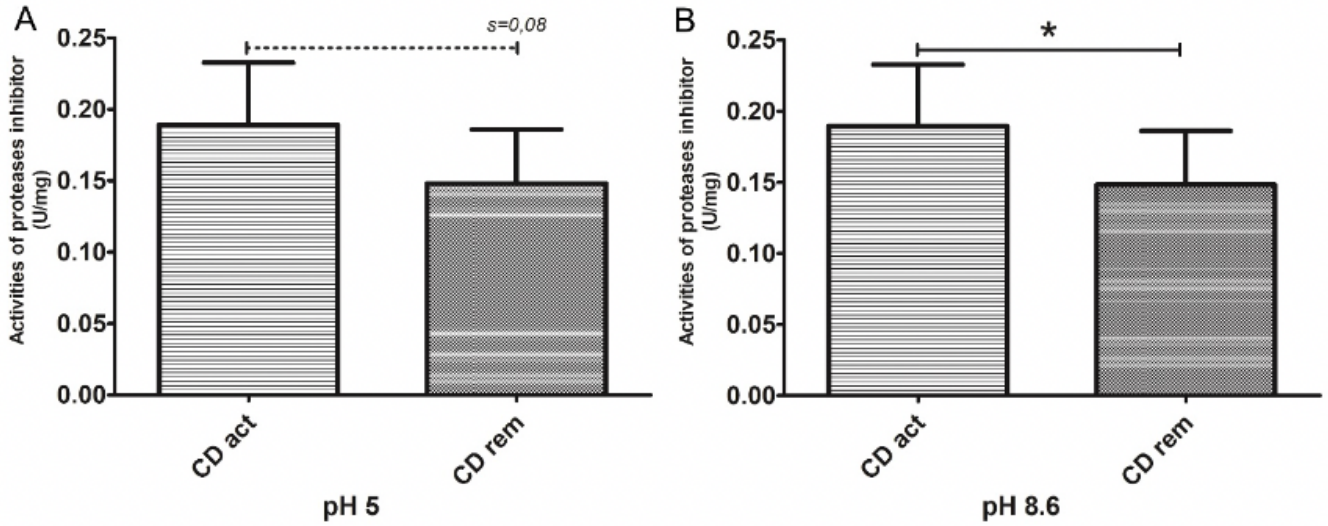

Figure 5

Activity of acid A (pH 5.0) and alkaline B (8.6) protease inhibitors in the plasma of patients with Crohn's disease – active (act) and in remission (rem) (±SD). The differences are statistically significant for comparisons between groups (act and rem) at $P \leq 0.05$.

In the group of patients with Crohn's disease treated biologically, acid protease activity (pH 5.0) was lower than in patients with Crohn's disease without biological treatment (Fig. 6).

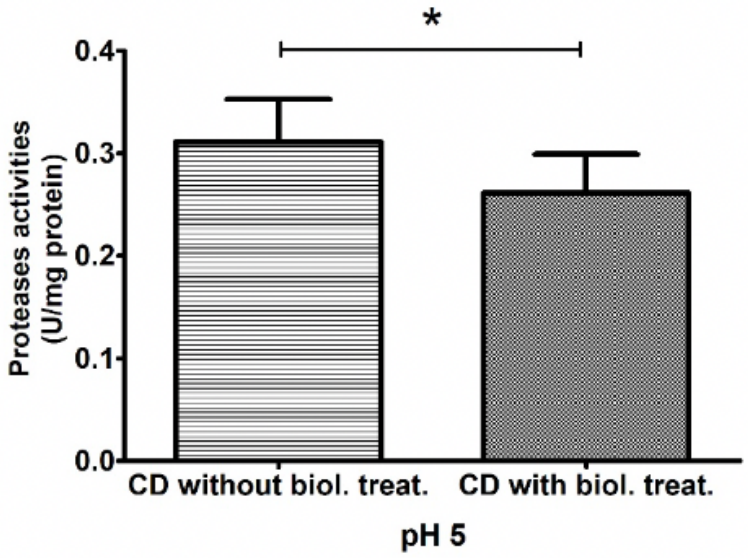

Figure 6

Activity of acid protease A (pH 5.0) in patients with and without biological treatment. The difference was statistically significant for comparisons between groups (with and without biological treatment) at $P \leq 0.05$. Other differences were statistically non-significant.

Activity of neutral (pH 7.0) and alkaline (pH 7.6 and 8.6) proteases in the plasma of patients with ulcerative colitis in remission was decreased in comparison to the active phase (Fig. 7).

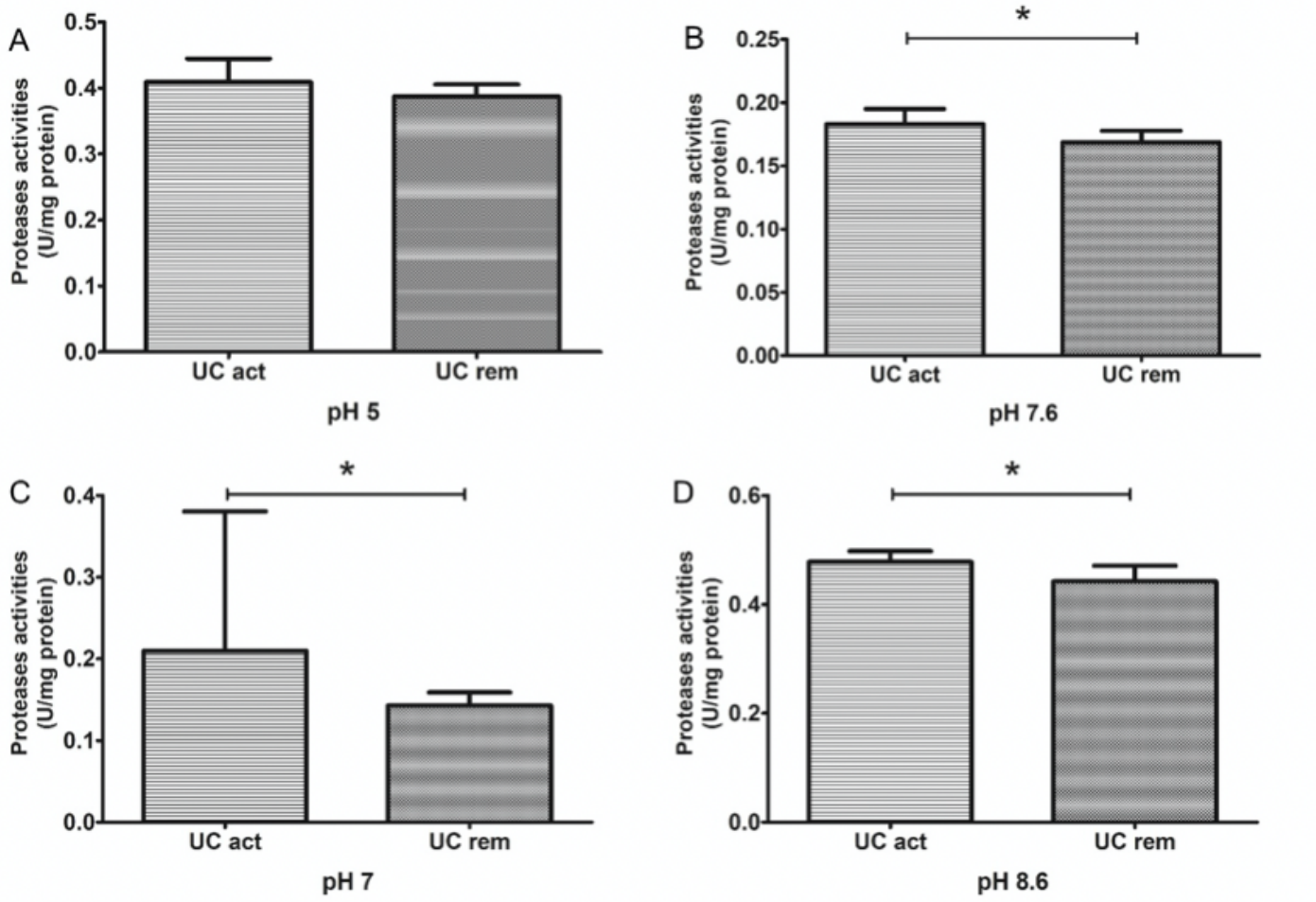

Figure 7

Activity of acid A (pH 5.0), neutral B (pH 7.0) and alkaline C and D (pH 7.6 and 8.6) proteases in the plasma of patients with UC – active (act) and in remission (rem) (±SD). The differences are statistically significant for comparisons between groups (act and rem) at $P \leq 0.05$.

The plasma activity of alkaline (pH 8.6) protease inhibitors was increased in patients with ulcerative colitis in the active phase compared to the group of patients in remission (Fig. 8).

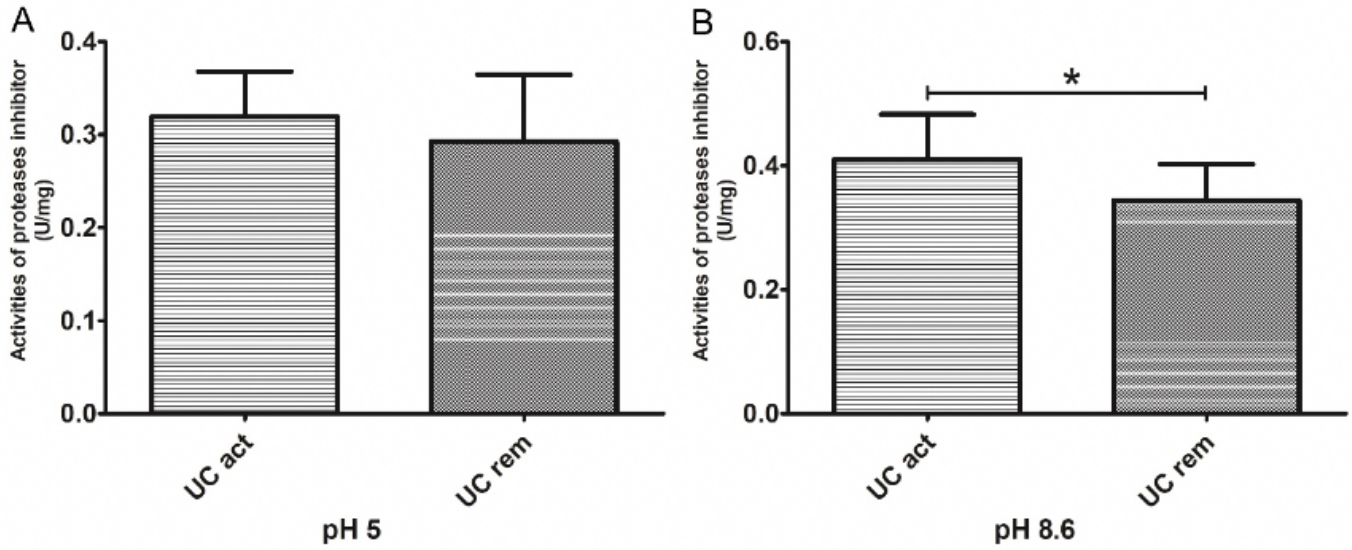

Figure 8

Activity of acid A (pH 5.0) and alkaline B (8.6) protease inhibitors in the plasma of patients with UC – active (act) and in remission (rem) (±SD). The differences are statistically significant for comparisons between groups (act rem) at $P \leq 0.05$.