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(54) Title: METHODS AND MEANS FOR PREVENTING OR TREATING INFLAMMATION OR PRURITIS

(57) Abstract

The invention relates to methods and means for preventing, treating or reducing inflammation by inhibiting proteolytic activity, more specifically for preventing or reducing inflammations of skin or intestine. The invention provides a method for reducing or preventing an inflammation comprising subjecting a mammal to a treatment with at least one inhibitor which is capable of inhibiting proteolytic activity. In a preferred embodiment of the invention, said inhibitor is a plant product, such as potato juice or an inhibitor derived thereof.

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Title: Methods and means for preventing or treating inflammation or pruritis

The invention relates to methods and means for preventing, treating or reducing inflammation by inhibiting proteolytic activity, more specifically for preventing or reducing inflammations of skin or intestine.

Inflammations of skin (dermatitis) or intestine (enteritis) are of various origin. Initially, allergic reactions, infections with (pathogenic) micro-organisms, excoriation by chemical or physical means, and other causes are instrumental in causing an inflammation. These causal events are immediately followed by the so necessary reaction of the body, resulting in an interplay of actions and events aiming at restoration of the skin or intestine in its original state. In this interplay of cause-and effect, various activities of proteolytic enzymes are seen. Granulocytes, mast-cells, macrophages and other immediate actors in inflammatory responses and attracted by cytokines to a site of inflammation, contain (and secrete) proteases, such as chymotryptic protease and elastase, that act as mediators or are instrumental in cleaving and removing proteins derived from pathogens or from the surrounding degenerated tissue. Bacteria, either as primary causal agent, or during a secondary infection, and other (pathogenic) micro-organisms, secrete proteases that damage the surrounding tissue for their purposes. In this battlefield between host and invader, excess proteolytic reactions are kept at bay by, often very specific, protease inhibitors. Well known are proteinase/proteinase inhibitor systems such as PMN-elastase/alpha-1-proteinase inhibitor and cathepsin G/alpha-1-antichymotrypsin.

Proteolytic enzymes in themselves, however, can also be a cause of inflammation. This is especially the case for digestive enzymes, which are found in the intestinal tract. In order to degrade dietary protein, the stomach, the pancreas and the small intestinal brush border secrete several kinds of proteases. Pepsin from the stomach works optimal at pH 2, pancreatic and brush border enzymes, such as trypsin, chymotrypsin and elastase work optimal at pH 7-8. In adults, the small intestine has a length of seven meters and the transit time of its contents is about 3 hours; this part of the intestine is colonised by only a few bacteria but is filled with a watery mixture of food and a wide array and large quantities of digestive enzymes, such as lipases and proteases. However, in the large intestine (colon and caecum) the water content is greatly reduced, and the activity of the enzymes is neutralised by i.e. bacteria. Neutralised and digested remnants of food and bacteria (faeces) finally leave the body via the rectum. Only when the colon cannot effectively reduce the water content and neutralise the enzymes, the faeces may still contain proteolytic activity, which, during periods of diarrhoea or faecal incontinence, may be very irritating to intra-anal and perineal skin.

The skin, especially of humans, is, although it is protected by the stratum corneum which consists mainly of keratine, as any other proteinaceous substance, very susceptible to the proteolytic action of proteases, consequently fluid like small intestinal content may cause severe inflammation.

In babies and infants, the intestine is much less well developed, especially the colon functions different from that in adults. This is the reason why digestive enzymes in faeces of babies and infants are not neutralized; the contents of faeces resemble more the contents of the small intestine, albeit having passed the colon. Therefore, perineal

(perianal) dermatitis is more often found with babies or infants than with adults. Also, (hospitalised) infants and children with gastro-intestinal disorders are prone to such a dermatitis Such a dermatitis or prunitis, defined by itchiness skin erythema, vesiculas, wetness, oedema or disruption (excoriation) of perineal skin, is also found with diaper rash, and can manifest itself in rather mild to very severe forms. With diaper rash, complicating factors are the accumulation of urine, whereby ureum is converted by faecal bacteria to ammonia, thereby raising the pH to an even better value for the activity of proteolytic enzymes. Since the skin is extremely susceptible to infections, care should be taken to prevent such inflammations related to (faecal) proteolytic activity.

Yet other cases of dermatitis are found with patients that have undergone resections of colon and/or ileum a stoma. Pouchitis, an intestinal inflammation, is a major complication of ileoanal anastomosis with reservoir construction after colon resection and is characterised by clinical symptoms and inflammation of the reservoir (pouch). Peristomal (circumstomal) dermatitis is found with those patients that have been provided with an ileostoma that opens up at the surface of the abdomen, ending in an artificial reservoir that needs to be emptied daily. In inflammatory bowel diseases (IBD, such as Crohn's disease (CD), ulcerative colitis (UC) and pouchitis), and inflammation with an unknown aetiology, the role of the intestinal flora and pathogens, proteolytic enzymes derived from these micro-organisms and endogenous (e.g. pancreatic or leukocyte/granulocyte) proteolytic enzymes and their contribution to degradation of protecting mucoglycoproteins and the underlying tissues is not understood.

Especially in above cases where the colon is removed or its function is affected or immature, it is evident that

the proteolytic activity is still very high when the faeces is excreted, leading to various degrees of perineal dermatitis.

It goes without saying that many medications and personal care items have been developed in order to remedy the (severly) itchy and often painful consequences of above discussed inflammations. General anti-inflammatory therapy often resorts to treatment with corticosteroids, despite the serious side-effects that are often seen with these medicaments. Other ways of treating are mainly based on providing either a protective layer to the skin, e.g by applying a lipid-based ointment, containing additives such as zinc, or by frequently cleaning an area at risk. Special personal care items have been developed, varying from specific wet wipes for perineal care, diapers that stay very dry despite heavy soiling by the child or patient, to products (stoma care applicances), such as adhesive and absorbing discs and stoma rinsing fluid, that are specifically designed for stoma care with patients with ileostomy or ileo-anal anastomosis.

However, none of these treatments can really do no more than alleviate one or more of above and below described clinical symptoms.

The invention provides a method for treating, reducing or preventing an inflammation or pruritis comprising subjecting a mammal to a treatment with at least one inhibitor which is capable of inhibiting proteolytic activity. Preferably, the invention provides a method where by a protease produced or secreted for example granulocytes , mastcells, macrophages and other actors in inflammatory processes is inhibited. The invention is applicable to human and veterinary medicine and care.

A preferred embodiment of the invention is wherein said mammal is a human suffering from for example dermatitis

or pruritis. Treating for example a dermatitis with a protease inhibitor reduces the proteolytic activity of the proteases involved in the inflammation pruritis. Especially when in the interplay of causes and effects seen during inflammation, the activity of proteolytic enzymes is too high, the invention provides a method to reduce this activity (be it from host or from invader) by treatment with at least one inhibitor which is capable of inhibiting proteolytic activity.

Said treatment is provided by applying said inhibitor in an ointment, cream, gel, powder, or any other suitable form to the location of the inflammation. These substances can for example also be carried on wipes impregnated with an inhibitor, in sprays or in rinsing fluid.

In a preferred embodiment of the invention, treatment is provided for an inflammation which is intestinal, perineal or peristomal, as is for instance seen with babies or infants with diaper rash, with children or adults with diarrhoea or faecal incontinence, with patients with inflammatory bowel syndrome and with stoma patients, which all suffer from the effects of proteolytic activity which is mainly faecal.

Treatment of faecal proteolytic activity can occur by applying said inhibitor in an ointment, cream, gel, powder, or any other suitable form to the perineal or peristomal location of the inflammation. Intestinal inflammations, such as seen with IBD or pouchitis can be treated by rinsing the affected location in the digestive tract by for example administering an enema, or can be administered orally, preferably in a pharmaceutical composition, such as a draught or mixture pill, that can passage relatively unaffected through oesophagus and stomach.

These inhibitor substances can for example also be carried on wipes impregnated with an inhibitor, in sprays or in rinsing fluid. Also, it is possible to impregnate a diaper

(during diaper production or shortly before use) with an inhibitor, thereby providing a method and means against diaper rash or pruritis. In a preferred embodiment, such a diaper is treated or impregnated with an inhibitor as provided by the invention in at least that diaper area (and underlying parts) that has, when in use, contact with the perineum of the baby, infant, child or adult. With diapers, said contact area normally comprises the diaper surface that is in contact with the perineum.

The invention provides a method of treatment which comprises administration to the patient or mammal prone to an inflammation of an inhibitor capable of inhibiting proteolytic activity of a protease. Inhibitors of proteolytic activity are widely known. For example, acid has an inhibiting effect on the hydrolysis of proteins by pancreatic proteases and thus a pH decreasing substance can be used as an inhibitor as provided by the invention.

Also, adsorbing substances such as activated charcoal (one such product is known as Norit), can act as protease inhibitor through their adsorbing properties. In the experimental part, several examples are given of a treatment provided by the invention whereby activated charcoal, for example Norit®, is used to treat an inflammation such as for example pouchitis.

In a preferred embodiment of the invention, the invention provides methods and means capable of inhibiting proteolytic activity of a protease. Many protease inhibitors are known (see for example G. Salvesen and H. Nagase. Proteolytic enzymes, a practical approach. Eds R.J. Beynon and J.S Bond In: The practical approach series. 1989). Although non-specific inhibitors are known (i.e. human plasma α-macroglobulin), most discriminate between protease classes or even subclasses. Substances such as peptide aldehydes or peptide chloromethyl ketones are very specific for subclasses

of proteases (proteinases), depending on the peptide sequence they mimic. Others, such as metal chelators act only against metallo-proteinases or calcium dependent proteinases. Class-specific inhibitors are found against serine proteinase, against cysteine proteinase, against aspartic proteinase, and so on. These protease inhibitors are often commercially available as purified substances for use in biochemical preparations and may be expensive.

A preferred method according to the invention is a method wherein an inhibitor is derived from a plant, i.e. said inhibitor is a plant product comprising protease inhibiting activity. As an example, such a product derived of a plant is activated charcoal, which is obtained by burning peat or wood. A much preferred method according to the invention is a method wherein an inhibitor is derived from a plant that can give rise to fruit, seed, tubers or roots. Derived herein for example comprises derived by (partial) purification or isolation or by obtaining the necessary genetic information and producing by modern recombinant technology known in the art.

Plants often protect their leaves, fruits, seeds, tubers or roots against pests by inclusion of potent protease inhibitors and mixtures thereof in those leaves, fruits, seeds, tubers or roots. For example, cereals and legumes, such as wheat or soy beans, contain protease inhibitors such as soy bean trypsin inhibitor (SBTI), which generally has activity against trypsine or chymotrypsin but not against other proteinase classes. Tubers and roots, such as potato and cassave, but also yam, beets and sweetroot, and others, contain potent inhibitors of a wide variety of digestive tract proteases such as aminopeptidases, carboxypeptidases, chymotrypsin, trypsin and elastase, and because of this broad range, tuber or root derived plant products comprising proteolytic activity according to the invention are

preferred. Potato tubers are an extraordinarily rich source of a variety of inhibitors of all major intestinal digestive endo- and exoproteinase of animals (Pearce et al., Arch. Biochem. Biophys, 213, 456-462, 1982). Such inhibitors act as anti-nutrients that are present as part of the natural chemical defense mechanisms of plants such as tubers and roots against attacking pests. In potatoes, major inhibitors are polypeptide trypsin inhibitor (PTI), polypeptide chymotrypsine inhibitor I and II (PCI-I and PCI-II), inhibitor II against chymotrypsin and trypsine, and carboxypeptidase inhibitor, which all have analogues in other plants. These act alone and in concert against the major animal digestive proteinases.

The invention provides the use of an inhibitor or plant product or extract capable of inhibiting proteolytic activity for preparing a pharmaceutical or personal care composition for reducing or preventing an inflammation or pruritis. In the experimental part an example is given of such a product which comprises potato juice or an inhibitor derived thereof, for example by freeze-drying. Such a composition can comprise an ointment, cream, gel, powder, or any other suitable form in which an inhibitor can be applied to a patient. In a preferred embodiment of the invention, the invention provides the use of an inhibitor or plant product capable of inhibiting proteolytic activity for preparing a composition for reducing or preventing an inflammation or pruritis which is an intestinal, perineal or peristomal inflammation or pruritis. Such a composition can be in the form of a rinsing fluid, can be contained in capsules that passage through oesophagus and stomach, can be in (prefabricated) wipes or diapers, wherein the inhibitor (or plant product) is added during production or shortly before use. In a preferred embodiment the invention provides the use of an inhibitor capable of inhibiting proteolytic activity

for preparing a personal or medical care composition for perineal (perianal) and/or peristomal care, for example to counter proteolytic activity that is faecal.

For example, it is possible to prevent perineal dermatitis by rinsing the reservoir and the perineal skin with a protease inhibitor containing fluid or a protecting ointment with protease inhibitors. Also, it is possible to pre-treat diapers or personal care compositions that adsorb soiling with inhibitor powder.

The invention provides the use of an inhibitor or plant product capable of inhibiting proteolytic activity for preparing a pharmaceutical or personal care composition wherein said inhibitor or product is derived from a plant, preferably wherein said plant can give rise to fruit, seed, tubers or roots, such as a potato plant. Such an inhibitor (product, composition or mixture), as explained above, is active against a protease which is selected from the group of pancreatic and granulocyte proteases. In a particular embodiment of the invention said inhibitor (composition or mixture) is capable of inhibiting papain and/or pronase, illustrating its broad spectrum and effectivity.

The invention also provides a pharmaceutical or personal care product (for example ointments, powder, fluids) comprising inhibitors of protease activity that is capable of for example preventing inflammation or pruritis caused by faeces (faecal proteases by inhibiting proteolytic enzymes from pancreatic and brush border origin; from bacterial (gutflora) origin; from leucocyte (granulocyte, mastcel, macrophage) origin in case of inflammation of the intestine; or

curing inflammation or pruritis caused by faeces by inhibiting proteases_(such as elastase, cathepsins) produced by tissue_macrophages, granulocytes, mastcells; or

curing skin inflammation, and other diseases in which inflammation and disease activity is related to infiltrating inflammatory cells (effectorcells) and the release of proteases; or

curing pruritis in general(treatment is often with antihistaminica) local application of ointments with protease inhibitor prevents histamine release from mastcells/protease release from fagocytes.

The invention also provides a personal care composition, rinsing fluids, wetties, powder, ointments, for peri-anal and/or peri-stomal care or a diaper comprising an inhibitor of proteolytic activity. The invention furthermore provides a protease inhibitor for use in a method according to the invention. Attention to skin care can already begin at the time of surgery, for example inhibitor containing rinse fluid. Inhibitors may be incorporated in stoma appliances, such as adhesive and absorbing discs and in stoma rinsing fluids and ointments.

Also, the invention provides a skin test for studying the effect of a protease inhibitor on proteolytic activity or inflammatory action of a substance, preferably of faeces.

The invention is further described in the experimental part which is not limiting the invention.

Experimental part

In adults, the small intestine has a length of seven meters and the transit time of its contents is about 3 hours; this is the reason why this part of the intestine is colonised by only a few bacteria, when compared to the large intestine. However the colon is colonized by large numbers of bacteria (10^{10} - 10^{11}/gram). The transit is slow (24 hours) and the main function of the colon is absorption of water.

Finally, faeces consists of one part solids and two parts of water. Half of the dry material are bacteria; the remnants are largely dietary fibre and host-derived material such as shed epithelial cells and mucus. The most active site of bacterial fermentation is the place where the contents of the ileum reaches the caecum. Abundant nutrients are available, the percentage water is high and the flora has optimal conditions to multiply. Few data about this part of the (human) intestine are known, but the pH, measured in sudden death victims, is very low (pH 4.5-5.5).

The colon flora consist for 99.9% of obligate anaerobic bacteria; anaerobic-facultative aerobic bacteria such as coliforms are a minority (about 10^4 - 10^7 bacteria/gram faeces). The anaerobic colon flora is very stable and it is nearly impossible to induce alterations at species or genus level, even by drastic changes in diet (antibiotics or infection with enteropathogens however might disturb the resident flora). One of the causes of this phenomenon is that the most important nutrients derive from endogenous material, digestive fluids, mucus, etc. A part of the digestive proteins (also the bile acids) are reabsorbed from the distal part of the ileum, the remainder is converted or digested in the colon. The colonflora is thought to play an important role in the inactivation of digestive pancreatic enzymes such as proteases.

In babies and infants, the intestine is much less well developed, especially the colon does not function as well as in adults. This is the reason why digestive enzymes in faeces of babies and infants are not neutralized and/or reabsorbed; its contents resemble more the contents of the small intestine, including a high proteolytic activity albeit having passed the colon.

The principal endogenous nutrient sources are probably glycoproteins from gastric and intestinal mucus which

contains up to 90% carbohydrate. Bacterial glycosidases degrade the oligosaccharide side chains which protect the glycoprotein from proteolytic destruction. When the protein core lacks the protection of the carbohydrates it is no longer resistant to proteolysis by pancreatic (and bacterial) proteases. In the healthy colon there is a balance between the production and the degradation of mucus.

Much attention has been paid to inflammatory bowel diseases (IBD): Crohn's disease (CD), ulcerative colitis (UC) and pouchitis. Pouchitis is a major complication of ileoanal anastomosis with reservoir construction, after colonresection for UC and is characterized by clinical symptoms and inflammation of the reservoir (pouch). The role of the intestinal flora in IBD was investigated concerning pathogens and their contribution to degradation of the protecting mucusglycoproteins.

Patients with inflammations in the gut show a loss of the integrity of the mucosa. We have studied the potential harmful role of bacterial glycosidases and bacterial and host-derived proteases by degrading mucus glycoproteins. Therefore in patients with IBD the composition of the intestinal flora and the activity of glycosidases and proteases was estimated. Also enzymatic activity was measured in germ-free rats to establish the influence of the flora.

These studies showed that faeces of patients with active CD, patients with an ileostomy and patients with a pouch have a high proteolytic activity. Proteases enter the duodenum largely as secretions from the liver, brush-border and pancreas. A part of the activity is lost in the terminal ileum, problably due to absorbtion and/or action of endogenous inhibitors. In faeces of healthy subjects only a very low or no enzyme activity at all, was estimated, which is probably largely of bacterial origin. However germ-free animals such as rats show a high proteolytic activity

throughout the whole large intestine. Patients with active IBD, ileostomy patients and patients with a pouch were found to have a high faecal proteolytic activity. From this we may conclude that a complete colonflora and a normal (slow) transit is necessary to inactivate these enzymes.

The high proteolytic activity in faeces of patients with IBD may cause an increased degradation of mucus glycoproteins and may play a role in the maintenance of the inflammation of the mucosa. In vitro experiments confirmed this hypothesis. The idea was born to treat patients such as those with an ileoanal anastomosis (IAA) with protease-inhibitors to prevent perineal dermatitis. Patients who are operated for UC or familial adenomatous polyposis (FAP), are considered for construction of an ileal reservoir after colon resection. This small reservoir is connected with the anus. The period after the operation is a hard time for most of the patients. Short after the operation the patients faeces has a watery consistence, the patients are often not (yet) continent and this results in irritation and pruritis of the perineal skin (perineal dermatitis). The major cause of perineal dermatitis is the degradation of the epidermis (which consists largely of the protein keratin) by proteases.

Proteolytic activity was measured in faeces of these patients and was found to be very high. Furthermore, 75% of the patients developed a moderate to severe perineal dermatitis; 25% did not have any sign of irritation.

Materials and methods

Proteolytic activity/subjects

Faecal samples from twenty-seven patients with Crohn`s disease (CD) were studied. Twelve patients, aged 27-58 years, had undergone intestinal surgery 3-12 years previously; locations of the resections were terminal ileum, ileum and

caecum, and colon. A second group of patients was not operated; the principal sites of inflammation were ileum, ileum and colon, and colon. The diagnosis CD was established with the usual clinical, radiological and histopathological critria. All patients were outpatients.

Twelve healthy volunteers, aged 23-48 years were examined for comparison.

Ileostomy effluents were obtained from five adult patients with a conventional ileostomy (aged 38-71 years). They had undergone total colectomy more than five years before, for relief of CD or ulcerative colitis (UC), and were all currently in good health.

Fourteen patients with a pouch (median age 27 years) were studied. The patients had a restorative proctocolectomy for UC or familial adenomatous polyposis. An S pouch was constructed in 12 patients, whereas in two patients a W pouch was created. This study was performed at least one year after the restorative colectomy. The diagnosis pouchitis was based on clinical symptoms, endoscopic features of acute non-specific inflammation and histological evidence of an inflammatory cell infiltrate. Using these criteria five patients presented pouchitis and nine did not (controls).

Faecal samples from thirteen patients operated for the construction of a reservoir with ileoanal anastomosis (IAA) were collected within 14 days after the operation. Proteolytic activity was measured in faeces from 31 healthy children, aged 4 months to 7 years.

Proteolytic activity/laboratory animals

Faeces from 4 conventional (Wistar) and 4 germ-free rats (Wag/Rij) were studied. From 2 conventional and 2 germ-free rats the contents of the intestinal tract were studied.

Faecal samples from 20 colectomized dogs, purebred Beagles (Harlan) were collected. Three ileostomy groups were

studied. In ten dogs a standard Brooke ileostomy was constructed by subtotal colectomy. In five dogs a valveless ileal reservoir (pouch) was fashioned by a side-to-side iso-antiperistaltic anastomosis. After a recovery period of 2 weeks a schedule of increasing periods of occlusion was started, except for 5 dogs. The maximum tolerable occlusion time was 2.5-3 h for the ileostomy group and 4-7 h for the reservoir group. In five dogs a continent ileostomy (Kock`s pouch) was constructed, which was emptied 2-5 times per 24 hours by catheterization.

Proteolytic activity/faecal samples and intestinal contents

Faeces was frozen and stored at -20°C within 3 h of passage. Preliminary studies showed no changes in proteolytic activity during at least 4 months of storage. Samples of 1 g were transferred to 24 vol of 0.1 M phosphate buffer pH 7.6 and homogenized ('Stomacher', Lab blender 400). Coarse particles were removed from the homogenates by gauze filtration (Utermohlen, refolded to 2 layers); these samples are further referred to as 'faecal homogenates'.

Immediately after killing the rats the whole intestine was removed and prepared. The small intestine was divided into 4 parts of equal length and the contents of each part was carefully washed with 0.1 M phosphate buffer (pH 7.2). Samples from coecum and colon were treated in the same way as faeces.

Proteolytic activity/macrophages

Mouse peritoneal macrophages (RAW) were cultured *in vitro* in 200 ml DMEM with 5 % FCS and 4mM glutamine and stimulated with 200 U TNFαmol medium. After 18 hours the cells were harvested, centrifuged and resuspended in 2 ml 0.1 M phosphate buffer pH 7.6 Total numbers of cells were about 3.10^8 per ml. The

cells were disrupted by repeated freezing and samples were used for protease assays.

Proteolytic activity/enzyme assay

Proteolytic activity was determined in the faecal homogenates in appropriate dilutions (up to 250-fold) in 0.1 M phosphate buffer (pH 7.6). Penicilline (0.1% w/v) was added to prevent bacterial growth. In the more recent inhibition tests no penicillin was used. Samples of 0.1 ml were incubated with 0.1 ml 1% (w/v) azocasein (Sigma) in phosphate buffer at 37°C during 1 h. The reaction was stopped by addition of 0.2 ml 10% (w/v) trichloroacetic acid (TCA); after 10 min at room temperature unhydrolysed azocasein, bacteria and other particles were removed by centrifugation at 10,000 rpm during 10 min. Then 0.1 ml of the clear supernatant was transferred to 0.1 ml of 1 N NaOH in flatbottom 24 wells microplates. To the blank assays azocasein was added after incubation an addition of TCA. The absorption of the samples was measured at 450 nm and compared with standard curves obtained from solutions of azocasein. Proteolytic activity was expressed as milligrams azocasein hydrolysed during 1 h per gram dry or wet weight of sample. Each diluted sample was tested for other than enzymatic substrate hydrolysis after heating at 80°C for 10 min. Spontaneous substrate hydrolysis was tested by incubation of the substrate with buffer.

N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-leucine-p nitroanilide (Sigma) was used as substrate for estimating purified human leucocyte elastase (Sigma) and elastase activity from mouse macrophages. Samples of 0.1 ml were incubated with 0.1 ml substrate (0.1% w/v) in 0.1 M phosphate buffer pH 7.6 in a flat-well microtiter plate. After 30 or 60 min the reaction was stopped by addition of 70 µl 30% acetic acid and the absorption was measured at 400 nm. One unit of

enzyme was defined as the amount wich released 1 µmol of p-nitoanilide per min at 37°C.

Proteolytic activity/effect of pH

To test the effect of pH on the proteolytic activity the faecal samples were diluted in citric acid-phosphate buffer (0.1 M $Na_2HPO_4/2H_2O$, 0.1 M citric acid/H_2O) pH 5.2, 5.8, 6.8 and 7.6. Additionally the substrate solutions were made in appropriate buffers.

Preparation of lectin-free potato proteins

Crude or relatively pure potato proteins were diluted in PBS. Human erythrocytes (disease-free) were added to potato proteins (endconcentration of the ery's 3%), carefully mixed for 1 min, centrifuged for 2 min at 1500 rpm. The supernatans was mixed again with the erythrocytes. This was repeated 5 times untill no haemagglutination was found in a haemagglutinationtest. The reciprocal value of the highest dilution of potato protein that showed definite haemagglutination was defined as the haemagglutinationtiter. The haemagglutinationtiter decreased from 25.600 to 25-1 for example. After lyophilizing, the inhibitoractivity of the lectins-free product was compared with the original protein fraction. No loss of inhibitor activity was found when tested in faecal samples with a high proteolytic activity and in purified protease solutions (trypsin, α-chymotrypsin and elastase, endconcentration 1%).

Furthermore lectins-free potato proteins are obtained by using for example chitooligo-agarose (Seikagaku).

Lectins from potato proteins are also inactivated, not by removing them from the protein solution, but by binding to solube carbohydrate moieties, such as for example N-acetochitooligosaccarides from hydrolized chitin and

glycopoteins from stomach or intestine. The lectines are still in the product but have lost their active site.

Proteolytic activity/protease inhibitors

The following inhibitors were used:

- Trasylol (Aprotinin) (Bayer) not diluted
- Ovomucoid () 1 % (w/v) in 0.1 M phosphate buffer pH 7.6
- Foetal Calf Serum (FCS) () not diluted
- Trypsin inhibitor II-from Soybean (STI) (T-9003; Sigma) 1% (w/v) in phosphate buffer pH 7.6
- Norit A (supra USP, 951191), B (Test EUR, A6910), E (Supra USP, 940260), PRSH, Carbomix, tablets
- Premium powder (Hollister)

Alternatively, potato juice (PJ) from "Bintjes" was prepared as follows. After peeling and washing the potatoes were smashed to pieces, filtered through cambric under addition of 0.2% ascorbic acid. The juice was centrifuged at 27.500 RCF for 30 min at 4°C, filtered through paper and again centrifuged. The clear yellow supernatans was filtered through a 0.45 micron filter and freeze-dried. This crude product was sterile (controlled with bloodagarplates) and contained about 25% protein. Ten gram PJ powder was derived of 200 ml juice.

- Potato juice (PJ) from "Bintjes" was prepared as follows. After peeling and washing the potatoes were smashed to pieces, filtered through cambric under addition of 0.2% ascorbic acid. The juice was centrifuged at 27.500 RCF for 30 min at 4° C, filtered through paper and again centrifuged. The clear yellow supernatans was filtered through a 0.45 micron filter and freeze-dried. This crude product was sterile (controlled with bloodagarplates) and contained about 25% protein. Ten gram PJ powder was derived of 200 ml juice.

In general, protease inhibitors which are present in potatoes for example can be recovered by grinding potatoes,

removing starch and other solids, and for example freeze-drying the juice.

The purity of the protease inhibitor preparation can be improved by removing non-proteinaceous material and/or low molecular weight peptides and/or amino acids present in potato juice by e.g. centrifugation, microfiltration, ultrafiltration, diafiltration or electrodialysis. Furthermore, protein can be selectively recovered in a relatively crude form from the potato juice matrix. This can be achieved by e.g. ultrafiltration, iso-electric precipitation, (co)flocculation with polyelectrolytes or any other flocculation aid, coprecipitation with other proteins, protein precipitation with salt (salting out), or by changing the quality of the solvent e.g. by adding aceton, methanol, ethanol or iso-propyl-alcohol, by iso-electric precipitation and thermal fractionation and other techniques known to anyone skilled in the art. Since protease inhibitors in potato juice are relatively heat stable, a moderate thermal treatment leads to denaturation and coagulation of less stable proteins. Coagulated protein can subsequently be removed by techniques as simple as e.g. centrifugation. Although some protease-inhibiting activity is lost, the purity of the remaining protease inhibitors is increased. Even further purification is possible by ultrafiltration or by salting out the protease inhibitors, and subsequent removal of salt and other undesired components by ultra- and diafiltration. Alternatively, isolation of several protease inhibitors is possible by affinity chromatography, either directly from the crude potato juice matrix or after pre-purification.

In most of the experiments the inhibitor was added to faeces (diluted 1:25 in buffer), mixed for 5-15 min and added to the substrate; PJ-powder was added to undiluted faeces (1:1) and after mixing, diluted (1:25) with buffer. In each

experiment controls were assayed (sterilized faeces, sterilized inhibitors, buffer solutions).

Proteolytic activity/purified enzymes

The following enzymes were tested in the inhibition experiments:

- bovine pancreatic trypsin (Serva)
- bovine pancreatic α-chymotrypsin (Merck, Sigma)
- bovine pancreatic elastase (Sigma)
- papaine (Sigma)
- pronase (Sigma)

(carboxypeptidase and leucinaminopeptidase were tested, but did not hydrolyze azocasein)

All enzymes were used in a concentration of 0.2 % (w/v) in buffer.

Proteolytic activity/skin tests

Skin tests were performed on the ventral part of the fore-arm. The following solutions were tested: 1. supernatant from faeces of a patient with an ieum reservoir with a high proteolytic activity; 2. the same supernatant, but sterilised; 3. supernatant with 0.25% STI (w/v) and 4. 0.25% STI in buffer. Two hundred µl of each solution were placed on folded cambric on the skin and covered with plastic and adhesion wound pad. Total incubation time was 7 h, but after 3 and 5 h 100 µl buffer was added to each of the test patches to prevent dehydration.

Inhibition of faecal proteolytic activity by products from Potato Juice Euro 1, Euro 2, Euro 3

Euro 1 is crude PJ powder, Euro 2 and 3 are more purified.

Materials and methods

Faecal samples

Faeces from 1 patient with an ileostomy, 1 patient with a good-functioning pouch, 1 patient 14 days after colectomy and the construction of a pouch, 2 babies aged 4 months were used.

Faeces were used undiluted except for the babies, which was diluted 1:1 in phosphate buffer pH 7.6 and centrifuged 10 min at 10,000 g.

EURO's

EURO's were used as 1:5, 1:10, 1:25, 1:50 and 1:100 dilutions in phosphate buffer pH 7.6.

Faeces and EURO were mixed 1:1 for 10 minutes, then the mixture was diluted in phosphate buffer pH 7.6 1:12.5.

In both dilutions proteolytic activity was measured with azocaseine as substrate.

Skin tests

Patch Test Chambers (van der Bend) of 10 by 10 mm, filed with 50 µl of a test solution were placed on the skin of the upper part of the back of 2 healthy subjects and fixed with Fixomull Stretch self adhesive tape; the distance between them was 15 mm. One series of 4 test chambers was placed from cranial to caudal, a second series from caudal to cranial.

The test solutions had the following composition:

A. elastase, trypsin and α-chymotrypsin, end concentration of each of the enzymes 1% (Enzyme Mix) soluted in sterilized faecal supernatant from an ileostomy patient (FS)

B. FS

C. Euro 2 (end concentration 5%) soluted in FS with Enzyme Mix

D. Euro 2 in FS

After 24 hours the test chambers were removed and the skin was rinsed with tap water. Sites were inspected for erythema and dermatitis after 1, 2, 4, 6 and 24 hours.

A comparable skintest was made with the more purified potato protein fraction (EURO 3), end concentration 1%. A fifth testchamber was placed to control contactdermatitis: E (potatoprotein in distilled water). Twelve healthy subjects were tested.

Allergy tests

Type 4 (contract dermatitis): 31 patients of the department of Dermatology (AZR) were tested with the relatively purified (Euro 3) potato protein according standard protocols.

Type 1 (IgE mediated): pricktests: 10 patients of the department of Allergy (AZR) with foodallergy were tested and 1 patient with a severe allergy towards potato protein.

Results

1 Proteolytic activity in faeces

Proteolytic activity in faeces from healthy subjects was low. However Table 1 shows that patients with CD, ileostomy patients and patients with a pouch (with and without pouchitis) have a high proteolytic activity.

Table 1: Proteolytic activity in faeces of healthy subjects and patients

	Proteolytic activity		Dry weight of faeces mg/g	
	median	(range)	median	(range)
Healthy subjects	17.9*	(7.5-44.0)	313	(164-403)
Patients with CD				
- no resections	47.9	(19.1-192.0)	216	(128-228)
- resections	228.7	(130.6-356.6)	134	(84-175)
Patients with ileostomy	336	(89-972)	88	(69-120)
Patients with a pouch				
- no pouchitis	14**	(5.5-23.5)	83	(57-103)
- pouchitis	14	(7.1-17.3)	52	30-110)
Patients with IAA	53	(18-105)	ND	(<30)

* azocasein hydrolyzed, mg/h/g dry faeces

** azocasein hydrolyzed, mg/h/g wet faeces

Comparable results were found in faecal samples of laboratory animals. Faeces from normal dogs and rats had a very low proteolytic activity. Proteolytic activity was found to be high in ileostomy output and in valveless pouches of dogs despite occlusion; however continent pouches showed a complete normalization concerning the proteolytic activity (and several other parameters which are not discussed in this context). In contrast with germ-free rats in the colon of conventional animals, the proteolytic activity is strongly decreased, which suggests a role for the colon flora in inactivation (and/or degradation) of digestive proteases. In infants, proteolytic activity varies with age. An estimate of

the proteolytic activity in faeces of healthy infants and children show in infants (n=10, 4-12 months) very high activity, in children (n=9, 1-2 years) lower, but still high activity and in children (n=12, 2-8 years) decreasing activity.

In a further experiment, the proteolytic activity in faeces of 31 children was again found to decrease with age, in children of 4 months (n=4): 191 mg hydrolyzed azocasein/h/g faeces, in children of 6 months (n=2):109 mg, in a child of 8 months (n=1):118 mg, in children of 11 months (n=3):105 mg, in children of 16 months (n=3): 73 mg, in children of 24 months (n=6): 34 mg, in children of 3 years (n=5): 24 mg, in children of 5 years (n=3): 3 mg, in children of 7 years (n=4): 14 mg was found.

2 Inhibition of proteolytic activity

pH

Figure 3 shows that the pH dependence of the proteolytic activity was similar in each of the tested samples. At pH 6.8 and 7.6 the activities were respectively three and four times higher than at pH 5.2 ($p<0.001$ for both comparisons).This means that at pH of 5.2 the proteolytic activity is inhibited for 75%.

STI

The next table (Table 2) shows the results of our first experiments with protease inhibitors. Conditions of the assays were different but Trasylol, ovomucoid and FCS had effects on the proteolytic activity which were less promising or (conflicting) than STI. In a concentration of 1% (w/v) the inhibition was more than 80%.

Table 2: Inhibition of proteolytic activity in patients and dogs

	% inhibition of the proteolytic activity					
	CD patients*		Ileostomy dog		pouch dog	
	n=4	n=1*	n=3°	n=1†	n=2+	n=1**
ovomucoid	68	93	84	52		
trasylol			54	56		16
STI 1% (0.25,0.5,0.75%)		93	84 (63,61, 45)			
FCS		94	0			
ovomucoid + trasylol			51			
ovo+tras+STI				76		
ovo+STI			70			
norit A				50		

* inhibitor added to faeces 1:2000 diluted; 20h incubated with substrate

° inhibitor added to faeces diluted 1:100; 2 h incubated

† undiluted faeces+ inhibitor (3+1), mixing for 2 h, then diluted 1:100

+ undiluted faeces +norit (4+1) mixing for 2h (or 13 min), then diluted 1:100

** 2 g faeces+ 0.5 ml trasylol; mixing for 15 min, then diluted

Norit

Several kinds of norit were tested with faeces from pouch patients with a high proteolytic activity for optimal adsorbing qualities, to be used as protease inhibitor in fluid to rinse IAA patients after their operation. In this experiment Premium powder was tested also. Table 3 shows that the adsorbing capacities of norit PRSH and norit E for proteases were extremely strong.

Table 3: Effect of norit on proteolytic activity in faeces

	% inhibition of the proteolytic activity*
Carbomix	0
Norit A (Serva)	83
Norit A	83
Norit B	0
Norit E	97
norit PRSH	100
norit tablets	58
premium powder	17

* faeces from 7 patients was diluted 1:25 with buffer with 5% norit; before addition of substrate the mixture was centrifuged (norit also may adsorb the substrate); values are medians.

Inhibition of the proteolytic activity by norit was confirmed by using skimmed milk plates; the caseine in the agar is hydrolyzed by proteases and clarification is seen after treatment with TCA.

Norit PRSH was tested in different concentrations at pH 5.2 and 7.6.

Table 4: Effect of Norit PRSH on proteolytic activity in faeces

	% Inhibition of the proteolytic activity	
	pH 5.2	ph 7.6
Norit PRSH 1%	76	36
2%	95	92
3-5%	100	100

Potato Juice (PJ)

PJ was initially prepared and tested as fluid; later on a freeze-dried product was prepared. The initial end concentration of the PJ powder in the faecal suspensions was 17%. Table 5 shows the inhibition of faecal proteolytic activity by PJ and PJ powder.

Table 5: Effect of PJ on proteolytic activity of faeces

	% Inhibition of the proteolytic activity			
Number of patients	4*	4°	7†	7+
PJ				
- undiluted	93	(50)		
- 1:5 diluted	90			
- 1:10 diluted	51			
- 1:25 diluted	23			
PJ powder				
- 17%(w/v)			88	97
- 10%			78	90
- 5%			53	74
- 2%			20	44

* faeces 1:12.5 diluted in buffer, then mixed with Pj(1+1)

° faeces and PJ 1:1 mixed for 15 min, centrifuged, 1:25 diluted

† faeces and PJ powder (1 g in 2 ml buffer) 1:1; this gives an end concentration of 17%; no further dilutions for the assay

+ same experiment as †, but the mixture was diluted 1:25 for the assay

Heating of the PJ powder in a solution of 1 g in 4 ml buffer (end concentration in faeces 5%) did decrease the inhibitor capacities as follows:

unheated PJ: 65% inhibition of the proteolytic activity
30 min at 55°C: 64%
30 min at 80°C: 47%
30 min at 90°C: 24%
30 min at 100°C: 14%

Effect of protease inhibitors on the activity of pure enzymes is shown in Table 6. PJ powder is a very potent inhibitor of several pancreatic enzymes, papain and pronase.

Table 6: Effect of protease inhibitors on purified enzymes

% Inhibition of the proteolytic activity					
	trypsin	chymotrypsin	elastase	pronase	papain
STI (0.125%)	99	99	15		
- STI-A	100	80	50	0	0
- STI-B	100	100	60	0	0
- STI-C	100	100	55	0	0
Trasylol (undil.)	95	95	10		
PJ*	100	100	100	38	83
PJ (30 min at 80°C					
- 1:5			100		
- 1:10	100	100	97		
- 1:30			95		
- 1:40			94		
- 1:50			91		
- 1:75			90		
- 1:100			88		
- 1:1000			54		

*1 g PJ powder was mixed with 2 ml and with 4 ml buffer

Testing of Trypsin Inhibitors from Soybeans

STI-A: STI-type I-S Sigma T 9003

STI-B: STI-type II-S Sigman T 9128

STI-C: Bowman-Birke Inhibitor Sigma T 9777

enzyme concentration was 0.02%, inhibitor concentration (end concentration) was 0.125%.

Possible interactions of the PJ-inhibitor with the substrate was tested by using different concentrations of azocasein in the same experiment. No interactions were found:

Table 7:

	% Inhibition of elastase (0.02%)-activity	
	1% azocasein	2% azocasein
PJ diluted:		
- 1:50	100	100
- 1:100	97	95
- 1:500	87	75
- 1:1000	65	66
- 1:2000	44	51

Skin tests

After removing the pads and the cambric the skin was carefully cleaned with tap water and judged immediately and after 1-18 h. No reaction was seen with sterilized faeces (2) nor with STI in buffer (4), however moderate redness, papulas and some vesiculas could be observed at location 1 (faecal supernatant). Location 3 (faecal supernatant with STI) showed a slight redness that disappeared within 60 min.

The effect of potato juice or inhibitors derived thereof was also tested in vitro and in a skin test. The results are shown in Figures 1-6. It is possible to inactivate 90-100% of total proteolytic activity, extracts

from potato, such as potato juice or inhibitors derived thereof are able to inactivate faecal proteases and this prevents inflammation.

In the skin test, PJ, and its various purified fractions were shown to be very effective when applied to treat and prevent an inflammation. Whereas as sterilized faecal supernatant from an ileostomy patient caused an inflammation of the skin, and a severe dermatitis (redness, oedema, vesiculas, pain) when proteolytic enzymes were added, no inflammation was found when potato juice inhibitor was added in both cases. For example ointments, such as creme or gels, when mixed with PJ inhibitor, are capable to inhibit or prevent the local dermatitis.

Furthermore, no allergic or other adverse reactions where observed against both the potato juice product.

Inhibition of macrophage proteases

The production of proteolytic enzymes by mouse macrophages was stimulated by TNFα. Addition of purified potato proteins (EURO 3) inhibited the activity of elastase-like proteases for 70%

Elastese activity in mU

Macrophages (6.10^8 cells)	26.5
Macrophages (6.10^8 cells) + EURO 3 (1%)	8.0

These results show that the activity of (puried) human leucocyte elastase is reduced by potato protease inhibitors

Discussion

Furthermore these experiments show that patients with intestinal inflammations and/or resections of the colon or ileum, but also infants and children up to 2 years of age

have a high faecal proteolytic activity. These enzymes are of pancreatic, brush-border, microbial and/or cellular (granulocytes, macrophages) origin. These enzymes impair the protective intestinal mucuslayer as well as the skin in the perineal zone.

Both crude and purified potato proteins (protease inhibitors) inhibit the activity of faecal proteases (hydrolyzing azocasein) and the activity of macrophage elastase (hydrolyzing N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-leucin-p nitroanilide. Purified pancreatic enzymes, trypsin, chymotrypsin and elastase are also inhibited by PJ(crude or purified)

In a skintest dermatitis developed within 24 h using purified pancreatic proteases dissolved in sterilized faecal supernatans. This test is microbiologically safe, but the additional effects of faecal compounds, such as bile acids are intact. Dermatititis was completely prevented by the addition of crude or purified potato protease inhibitors to the testsolution.

It is probably not wise to use faecal supernatans again (for reasons of safety), but a mixture of the 3 purified enzymes in appropriate concentrations has the same effect. Azocasein is a substrate which is hydrolysed by several hydrolytic enzymes, but also enzyme specific substrates can be tested. STI is just one of the inhibitors from soybeans, inhibiting trypsin and chymotrypsin, but not elastase.

FIGURES

Figure 1: Inhibition of faecal proteolytic activity by products from potato juice.

Faeces from 1 patient with a well functioning pouch was used.

Faeces was used undiluted.

EURO's were used as 1:5, 1:10, 1:25, 1:50 and 1:100 dilutions in phosphate buffer pH 7.6.

Faeces and EURO were mixed 1:1 for 10 minutes, then the mixture was diluted in phosphate buffer pH 7.6 1:12.5.

In both dilutions proteolytic activity was measured with azocaseine as substrate.

Figure 2: Inhibition of faecal proteolytic activity by products from potato juice

Faeces from 1 patient with an ileostomy was used.

Faeces were used undiluted.

EURO's were used as 1:5, 1:10, 1:25, 1:50 and 1:100 dilutions in phosphate buffer pH 7.6.

Faeces and EURO were mixed 1:1 for 10 minutes, then the mixture was diluted in phosphate buffer pH 7.6 1:12.5.

In both dilutions activity was measured with azocaseine as substrate.

Figure 3: Inhibition of faecal proteolytic activity by products from potato juice

Faeces from 1 patient 14 days after colectomy was used.

Faeces were used undiluted.

EURO's were used as 1:5, 1:10, 1:25, 1:50 and 1:100 dilutions in phosphate buffer pH 7.6.

Faeces and EURO were mixed 1:1 for 10 minutes, then the mixture was diluted in phosphate buffer pH 7.6 1:12.5.

In both dilutions proteolytic activity was measured with azocaseine as substrate.

Figure 4 and Figure 5: Inhibition of faecal proteolytic activity by products from potato juice

Faeces from 2 babies aged 4 months were used.

Faeces were used diluted 1:1 in phosphate buffer pH 7.6 and centrifuged 10 minutes at 10,000 g.

EURO's were used as 1:5, 1:10, 1:25, 1:50 and 1:100 dilutions in phosphate buffer pH 7.6.

Faeces and EURO were mixed 1:1 for 10 minutes, then the mixture was diluted in phosphate buffer pH 7.6 1:12.5.

In both dilutions proteolytic activity was measured with azocaseine as substrate.

Figure 6: Patch Test Chambers (van der Bend) of 10 by 10 mm, filed with 50 µl of a test solution were placed on the skin of the upper part of the back of 2 healthy subjects and fixed with Fixomull Stretch self adhesive tape; the distance between them was 15 mm. One series of 4 testchambers was placed from cranial to caudal, a second series from caudal to cranial.

The test solutions had the following composition:

A. elastase, trypsin and α-chymotrypsin, end concentration of each of the enzymes 1% (Enzyme Mix) soluted in sterilized faecal supernatant from an ileostomy patient (FS)
B. FS
C. Euro 2 (end concentration 5%) soluted in FS with Enzyme Mix
D. Euro 2 in FS

After 24 hours the test chambers were removed and the skin was rinsed with tap water. Sites were inspected for erythema and dermatitis after 1, 2, 4, 6 and 24 hours.

Figure 7: The same patchtest as described under figure 6, but the crude inhibitorfraction was replaced by the more purified fraction (EURO 3).E.EURO 3 in distilled water.

Claims

1. Use of an inhibitor capable of inhibiting proteolytic activity for preparing a pharmaceutical composition for reducing or preventing an inflammation.

2. Use according to claim 1 wherein said inflammation is an intestinal, perineal, peri-anal or peristomal inflammation.

3. Use of an inhibitor capable of inhibiting proteolytic activity for preparing a personal care composition for peri-anal and/or peri-stomal care.

4. Use of an inhibitor capable of inhibiting proteolytic activity for preparing a diaper.

5. Use according to anyone of claims 1 to 4 wherein said proteolytic activity is faecal.

6. Use according to any one of claims 1 to 4 wherein said inhibitor is derived from a plant, preferably wherein said plant can give rise to fruit, seed, tubers or roots.

7. Use according to claim 6 wherein said plant is a potato plant.

8. Use according to any of claims 1 to 4 wherein said inhibitor is capable of inhibiting a protease which is selected from the group of pancreatic and granulocyte proteases.

9. Use according to claim 8, wherein said inhibitor is at least capable of inhibiting papain and/or pronase.

10. A personal care composition for perineal and/or peristomal care or a diaper comprising an inhibitor of proteolytic activity.

11. A method for reducing or preventing an inflammation comprising subjecting a mammal to a treatment with at least one inhibitor which is capable of inhibiting proteolytic activity of a protease.

12. A method according to claim 11 wherein said mammal is a human being.

13. A method according to claim 11 or 12 wherein said inflammation is an intestinal, perineal, peri-anal or peristomal inflammation.

14. A method according to any of claims 11 to 13 wherein said proteolytic activity is of faecal origin.

15. A method according to any of claims 11 to 14 wherein said inhibitor is derived from a plant.

16. A method according to claim 15 wherein said plant can give rise to fruit, seed, tubers or roots.

17. A method according to claim 16 wherein said plant is a potato plant.

18. A protease inhibitor for use in a method according to anyone of claims 11 to 17.

19. A plant product comprising protease inhibiting activity for use in a method according to anyone of claims 11 to 17.

20. A plant product according to claim 19 wherein said plant is potato juice and/or wherein said product comprises potato juice or an inhibitor derived thereof.

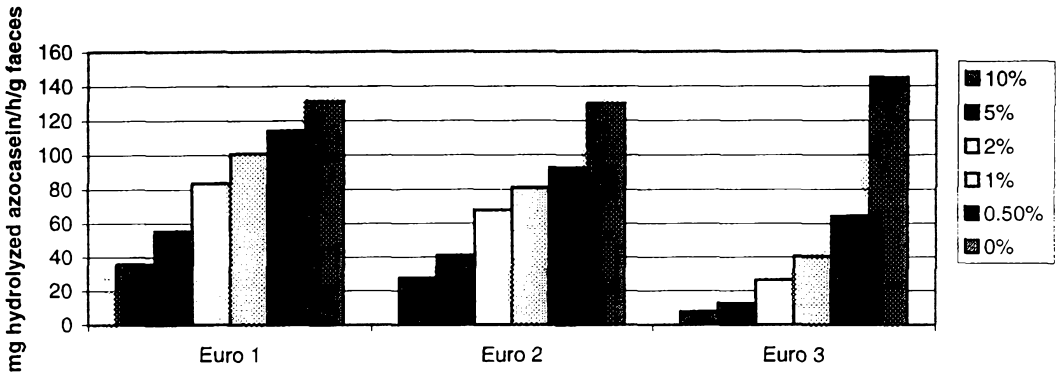
21. A personal care composition or diaper comprising a plant product according to claims 19 or 20.

22. A skin test for studying the effect of an inhibitor of proteolytic activity on inflammatory action of a substance, preferably of faeces, comprising sterilizing faeces and adding at least one proteolitic enzyme to said sterilized faeces, further comprising placing a sample of said faeces to the skin of a healthy subject.

23. A skin test according to claim 22 wherein said faeces is derived from an infant or a patient.

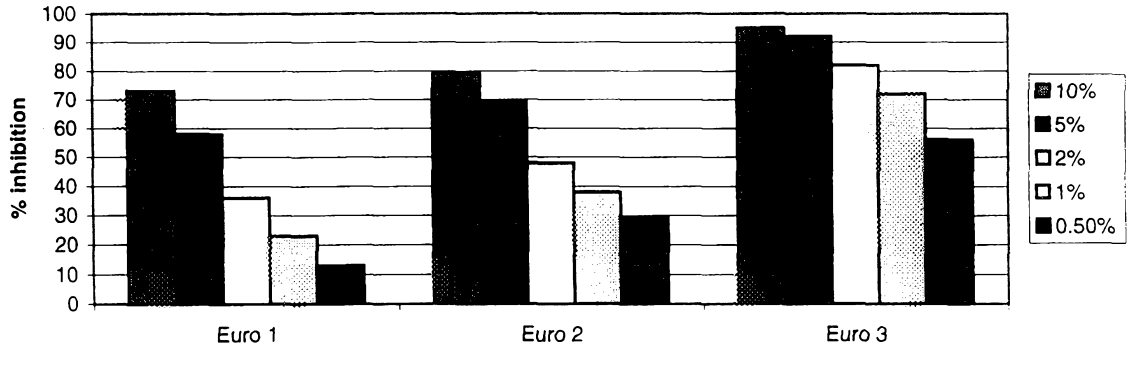
	Euro 1	Euro 2	Euro 3
10%	35.7	27.4	7.5
5%	55.2	40.9	12.3
2%	83.5	67.7	26.3
1%	100.8	80.8	40.3
0.50%	114.1	92.4	64
0%	131.2	129.9	144.7

Fig 1a. Effect of EURO on faecal proteolytic activity. Patient with pouch (F)



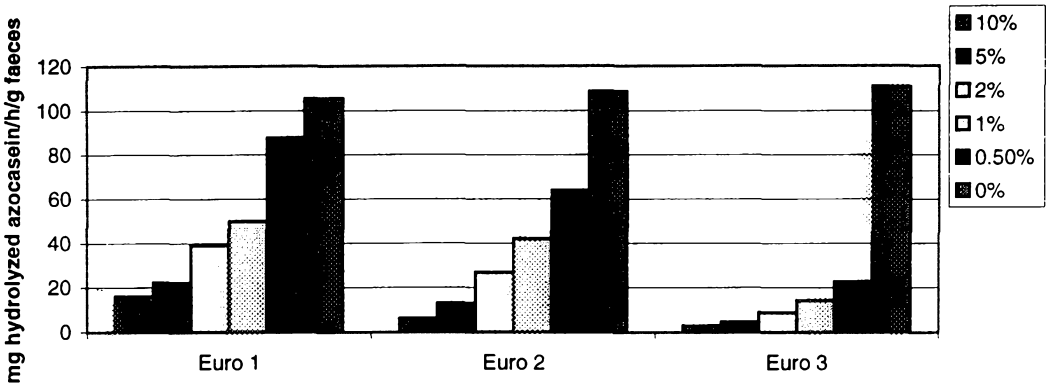
	Euro 1	Euro 2	Euro 3
10%	73	79	95
5%	58	69	92
2%	36	48	82
1%	23	38	72
0.50%	13	29	56

Fig 1b. Effect of EURO on faecal proteolytic activity. Patient with pouch (F)



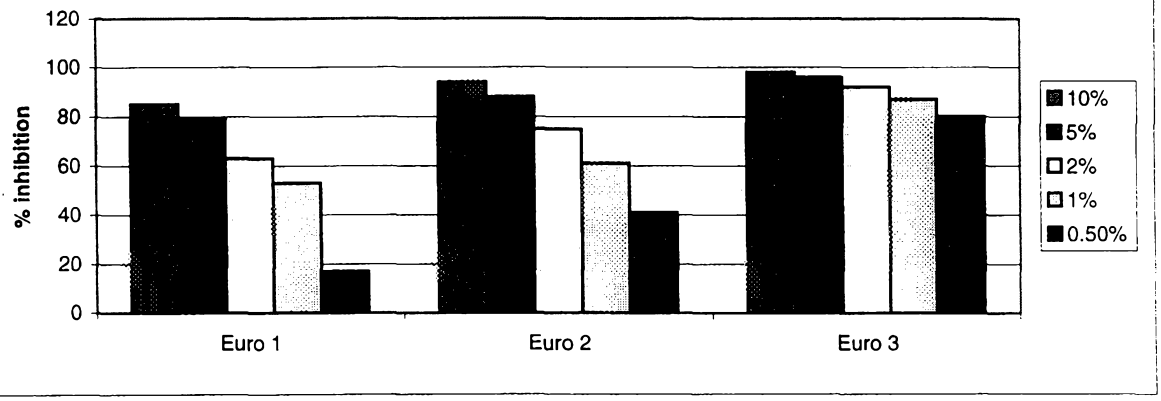
	Euro 1	Euro 2	Euro 3
10%	15.9	6	2.7
5%	22	12.9	4.5
2%	39	26.8	8.7
1%	49.9	42	14.2
0.50%	87.7	63.8	22.6
0%	105.4	108.5	110.9

Fig 2a. Effect of EURO on faecal proteolytic activity. Patient with stoma (F)



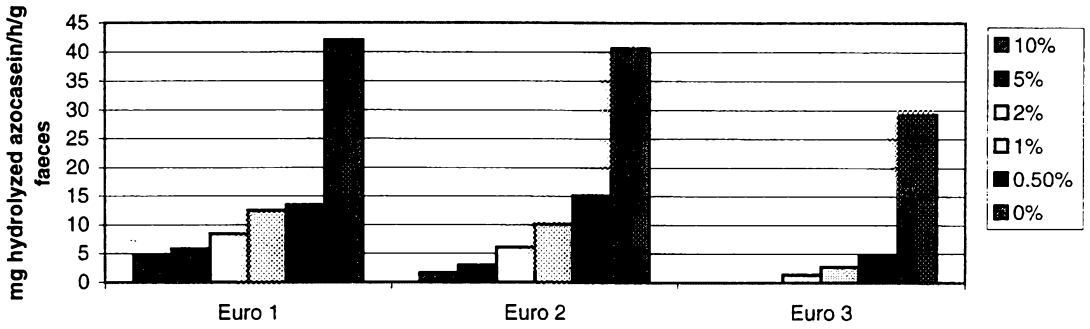
	Euro 1	Euro 2	Euro 3
10%	85	94	98
5%	79	88	96
2%	63	75	92
1%	53	61	87
0.50%	17	41	80

Fig 2b. Effect of EURO on faecal proteolytic activity. Patient with stoma (F)



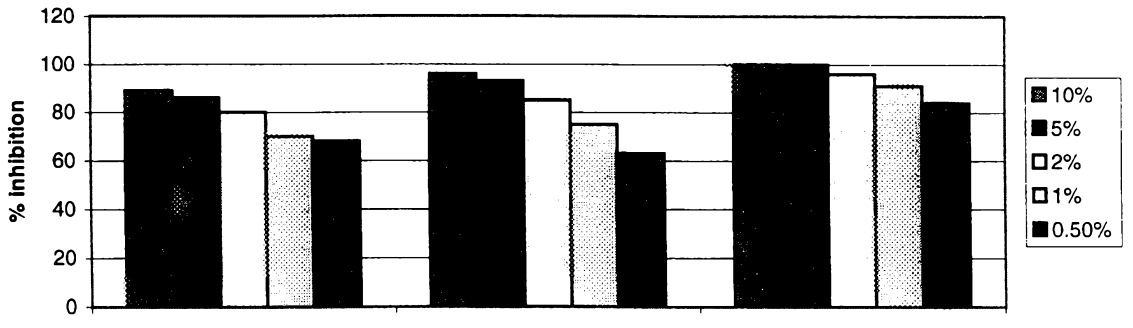
	Euro 1	Euro 2	Euro 3
10%	4.6	1.6	0
5%	5.8	2.9	0
2%	8.4	6.1	1.3
1%	12.5	10.1	2.7
0.50%	13.4	15.1	4.7
0%	42	40.6	29.2

Fig 3a. Effect of EURO on faecal proteolytic activity. Patient (R) with I.A.A. (Two weeks after operation).



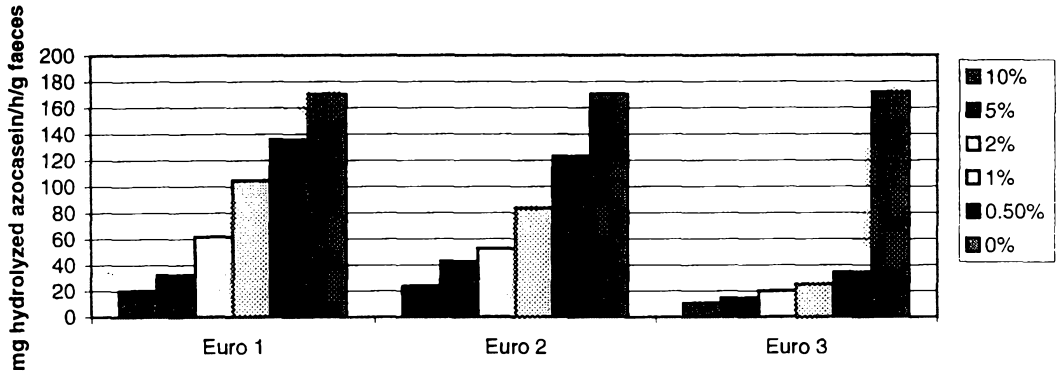
	Euro 1	Euro 2	Euro 3
10%	89	96	100
5%	86	93	100
2%	80	85	96
1%	70	75	91
0.50%	68	63	84

Fig 3b. Effect of EURO on faecal proteolytic activity. Patient (R) with I.A.A. (Two weeks after operation).



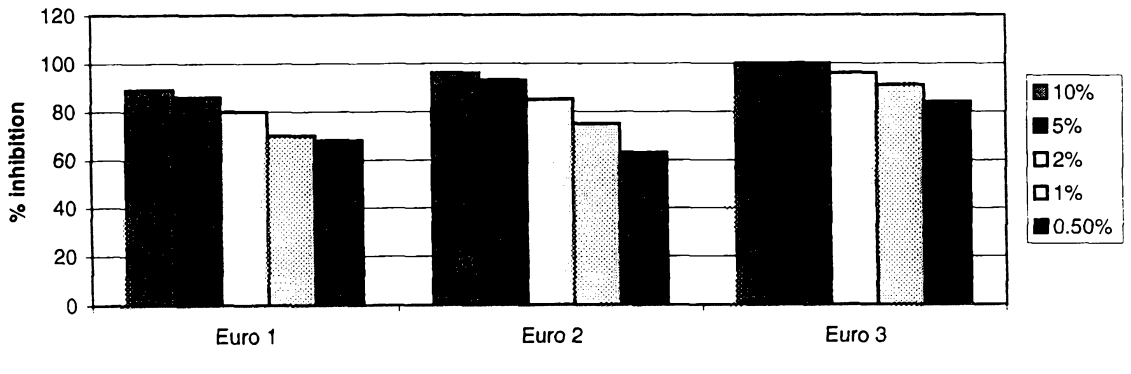
	Euro 1	Euro 2	Euro 3
10%	20.1	23.7	10.5
5%	32.3	42.7	14.5
2%	61.7	52.6	19.9
1%	104.6	83.3	25.2
0.50%	135.6	122.9	34.3
0%	170.3	170.3	171.8

Fig 4a. Effect of EURO on faecal proteolytic activity. Baby 1 (4 months).



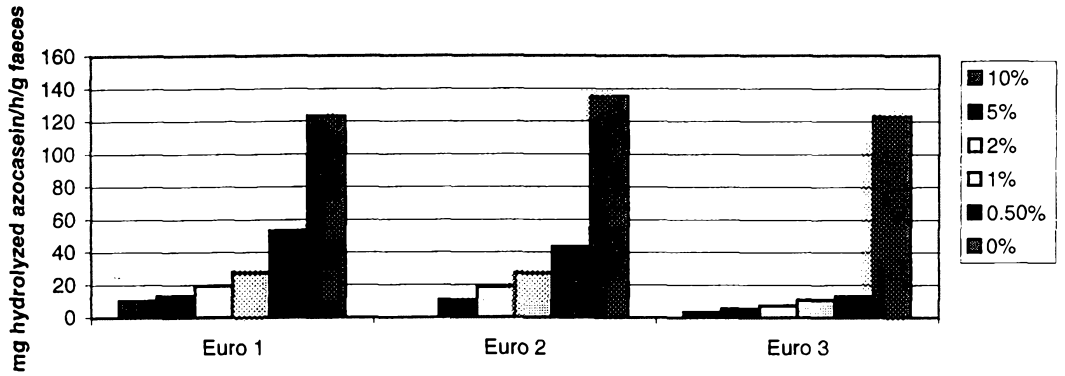
	Euro 1	Euro 2	Euro 3
10%	88	86	94
5%	81	75	92
2%	64	69	88
1%	39	51	85
0.50%	20	28	80

Fig 4b. Effect of EURO on faecal proteolytic activity. Baby 1 (4 months).



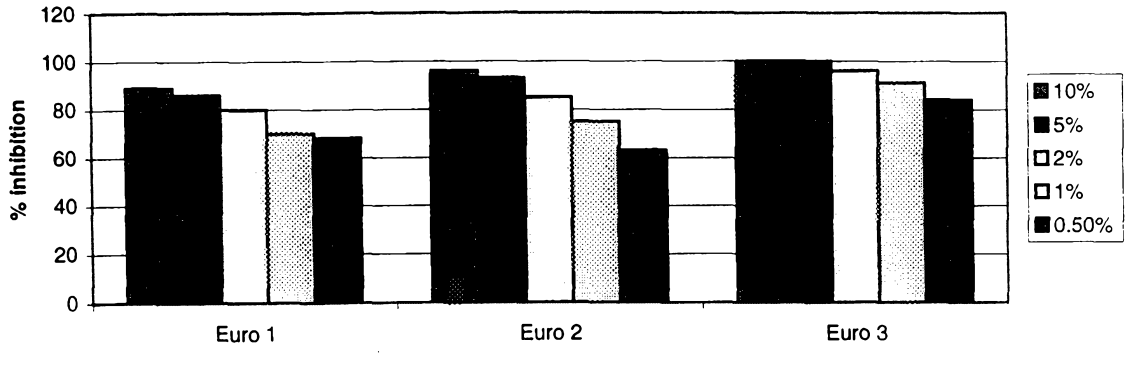
	Euro 1	Euro 2	Euro 3
10%	10.2	0	3.1
5%	13	10.5	5.2
2%	19.4	18.6	7.2
1%	27.5	27.2	10.7
0.50%	53.1	43.2	12.8
0%	123.1	135.1	123.1

Fig 5a. Effect of EURO on faecal proteolytic activity. Baby 14 (4 months).



	Euro 1	Euro 2	Euro 3
10%	92	100	97
5%	89	92	96
2%	84	86	94
1%	78	80	91
0.50%	57	68	90

Fig 5b. Effect of EURO on faecal proteolytic activity. Baby 14 (4 months).



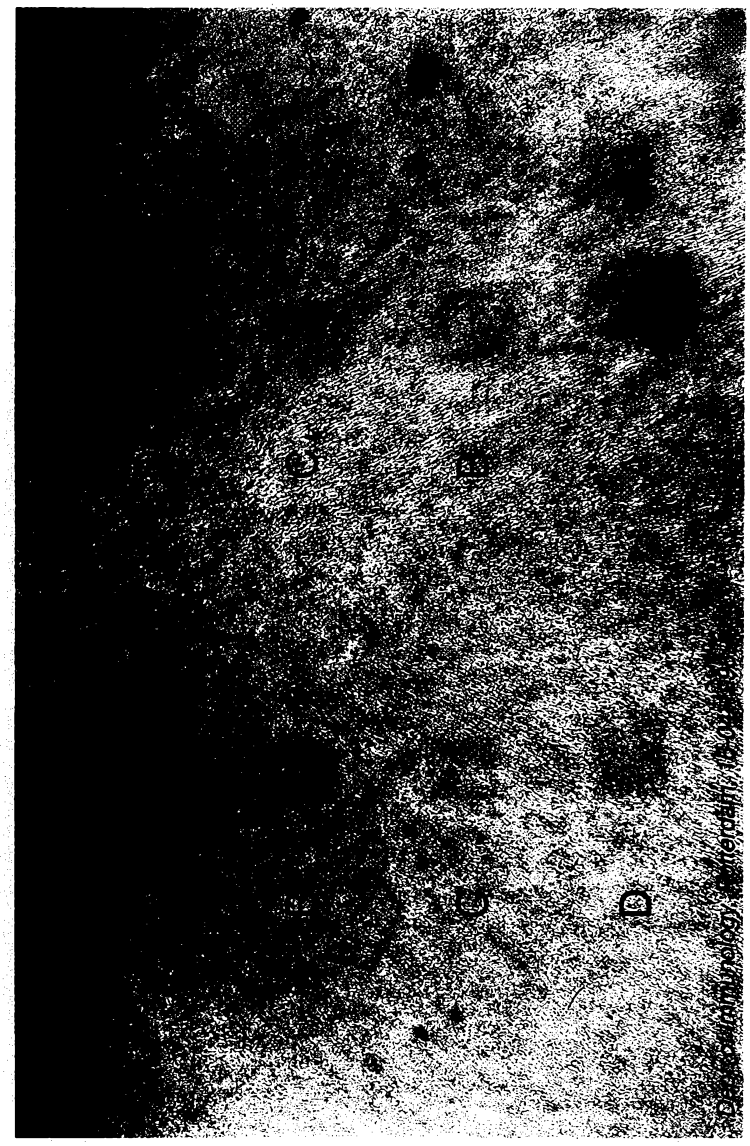
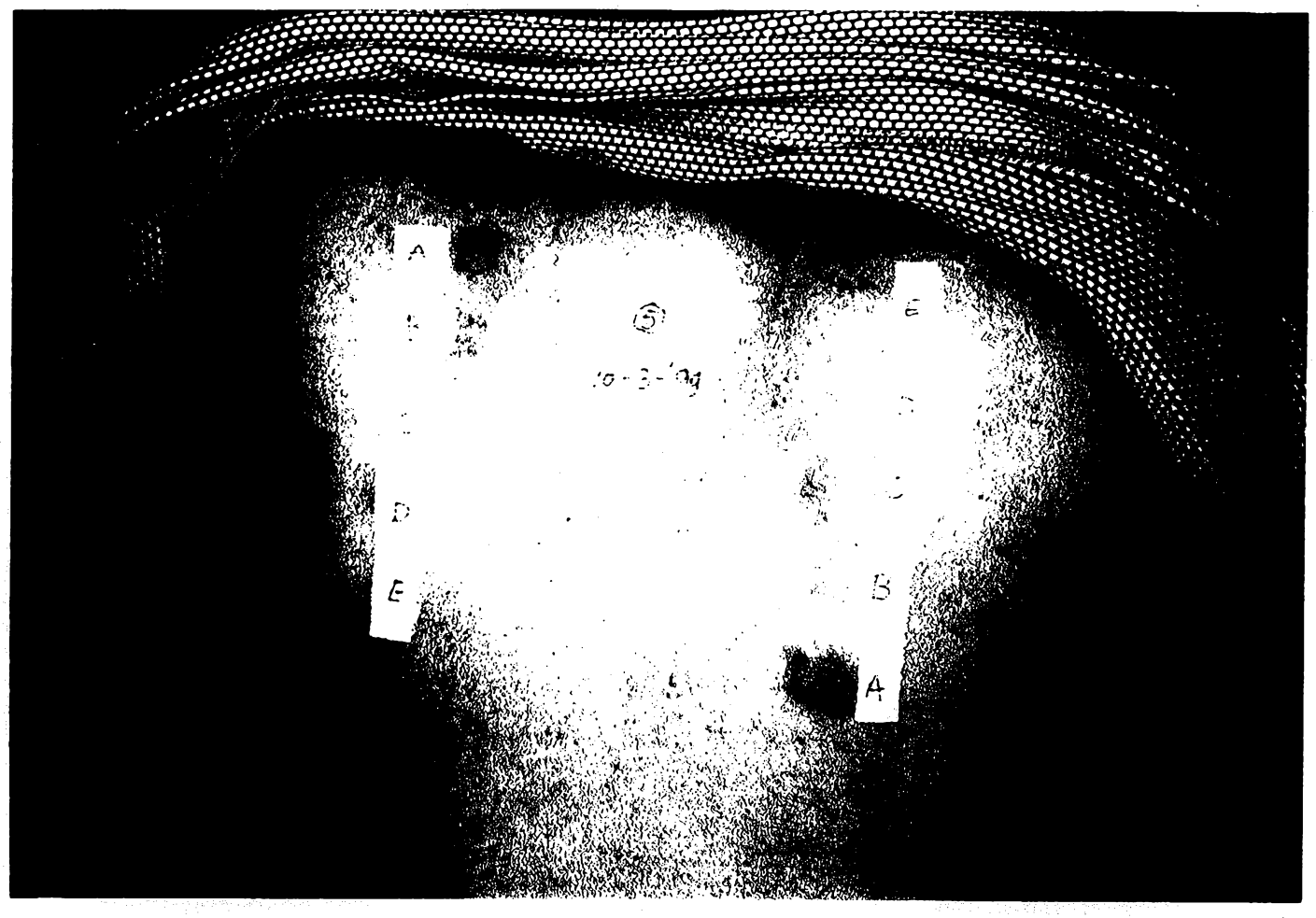
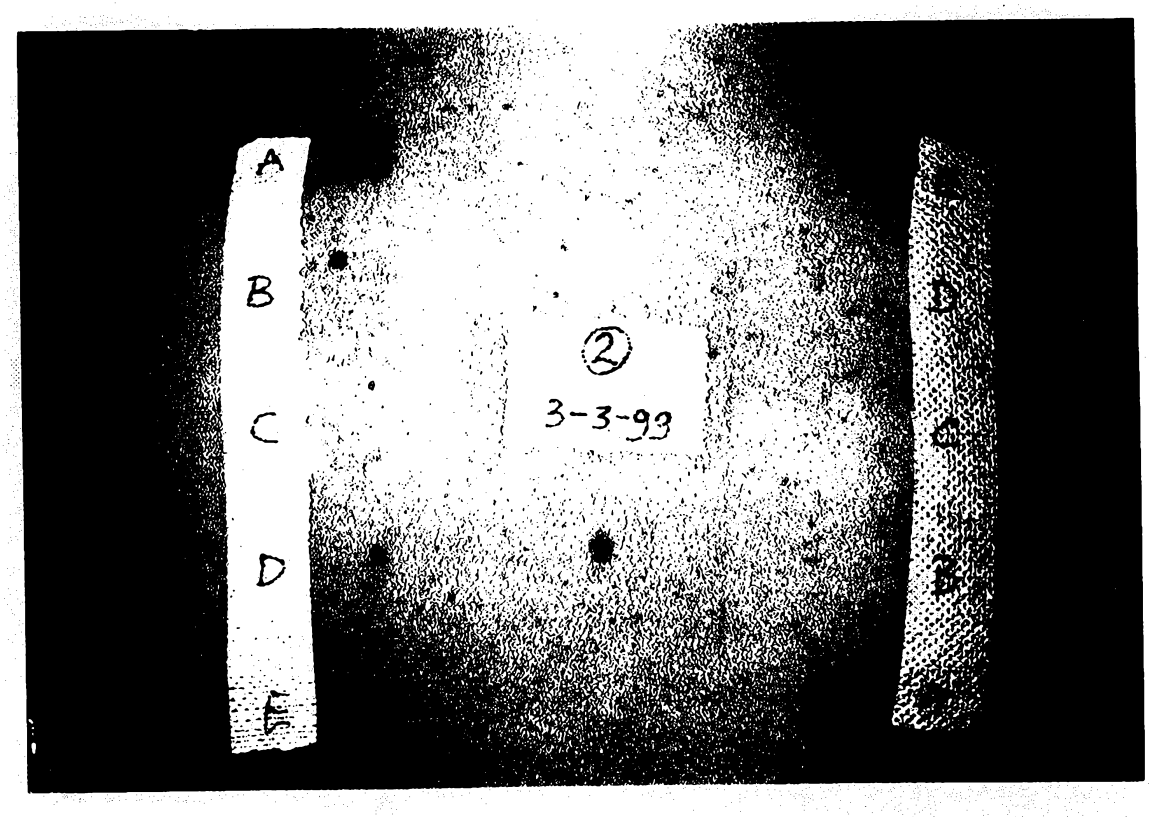


Fig. 6


A
E
D
E
B
A


A
B
C
D
E
3-3-99