Mast cell tryptase, a potent inflammatory mediator, requires heparin for enzymatic activation

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SAMMANFATTNING

Mastcellstryptas är ett trypsinliknande proteas som lagras i komplex med heparinproteoglykan eller kondroitinsulfatproteoglykan i mastcellens granula. Tryptas är en viktig inflammationsmediator vid bland annat allergiska reaktioner och astma. I denna studie har vi undersökt förutsättningarna för aktivering av humant βI-tryptas och βII-tryptas, vilka är de dominerande typerna av tryptas i humana mastceller. βI-tryptas och βII-tryptas skiljer sig åt strukturellt med endast en aminosyra, nummer 102, där βI-tryptas har asparagin (Asn) som tillika är en glykosyleringsplats och βII-tryptas har lysin (Lys). Detta betyder att βI-tryptas är glykosylerat medan βII-tryptas saknar glykosylering på den positionen. Vi har visat att både βI-tryptas och βII-tryptas är beroende av heparin för aktivering och att optimal aktivitetsnivå uppnåddes vid surt pH för βI-tryptas medan βII-tryptas inte är lika pH-beroende. Både βI-tryptas och βII-tryptas har stark affinitet för heparin-Sepharose vid surt pH, denna affinitet minskar vid neutralt pH. βI-tryptas och βII-tryptas visade båda en klockformad dos-responskurva för heparin-inducerad aktivering. Resultaten från denna studie visar, sammantaget med resultat från en parallell studie, att heparin har en betydande roll för aktivering av humant β-tryptas.

ABSTRACT

Mast cell tryptase, a tetrameric serine protease stored in mast cell granules in complex with heparin proteoglycan or chondroitin sulphate proteoglycan, is an important effector molecule in inflammatory reactions like allergies and asthma. In this study we have investigated the requirements for activation of human βI-tryptase and βII-tryptase, the major tryptases of human mast cells. βI-tryptase and βII-tryptase differ in only one amino acid, no. 102, where βI-tryptase has an asparagine (Asn) residue which is also site for glycosylation while βII-tryptase has a lysine (Lys) residue and lacks glycosylation at that site. We found that both βI-tryptase and βII-tryptase were dependent on heparin for activation and that optimal activity for βI-tryptase occurred at acidic pH while activation of βII-tryptase was less pH-dependent. Both βI-tryptase and βII-tryptase had a strong affinity for heparin-Sepharose at acidic pH but this affinity decreased at neutral pH. The β-tryptases both showed a bell shaped dose response curve for heparin induced activity. These results, taken together with results from a parallel study indicate an important role for heparin in the activation of human β-tryptase.

INTRODUCTION

The immune system protects the body from pathogens for example bacteria, viruses and parasites. The immune response is divided into two major groups, the innate (non specific) immune response and the adaptive immune response. The innate response occurs as a first line of defence when exposed to a pathogen and is executed mainly by phagocytic cells such as monocytes, macrophages and polymorphonuclear neutrophils. The adaptive immune response is primarily based on the actions of lymphocytes, mainly B-cells and T-cells, and is highly specific for a particular pathogen. Several other cell types including basophils, mast cells and platelets are also involved in combating infection by mediating inflammation in the affected area. The purpose of inflammation is to increase the concentration of cells participating in an immune response at the site of the infection. This is accomplished by increasing the blood flow to the area, increasing capillary permeability to allow for larger molecules than normal to pass from the vessels to the surrounding tissue and by facilitating migration of leukocytes into the infected area by means of chemotaxis.

When the immune system fails to function normally different problems can occur. Autoimmunity is a result of the immune system failing to recognize the body's own tissue, interpreting the tissue as foreign and mounts a response towards it. This occurs for instance in rheumatoid arthritis. If parts of the immune system fails it may be unable to generate a sufficient response to an infection, this is called immunodeficiency and can be hereditary or acquired. Yet another case is when the immune system mounts a reaction that is unproportional to the potential harm of the antigen, or a reaction is exerted after exposure to a harmless antigen. This is referred to as hypersensitivity and in these cases the immune response itself can be more damaging than the antigen the body is exposed to. Allergic reactions are examples of hypersensitivity. Hypersensitivity is divided into four different types. A type I reaction is when an allergen cross-links two IgE molecules bound to the surface of mast cells causing degranulation and subsequent release of inflammatory mediators that produce allergic reactions. Type II reaction is an antibody mediated reaction where antibodies of either IgM or IgG type are produced to interact with surface antigen on the target cell. The target cell can be either the body's own cells or foreign antigen such as for example transfused blood cells. The labelling with antibodies on these cells will subsequently lead to destruction of the cells by the immune system. Type III reaction is generated when antigen-antibody complexes form at a rate that cannot be sufficiently cleared by the body. Accumulation of these immune complexes in tissues causes inflammation and tissue damage. Type IV reactions occur as a result of specific T cells responding to an antigen, resulting in release of cytokines which in turn promotes release of inflammatory mediators from macrophages. Examples of type IV reactions are contact allergies (Roitt *et al.*, 2001).

Mast cells

Mast cells (MCs) originate from haematopoietic stem cells in the bone marrow and are released to the circulation as immature cells. The MCs mature in different tissues under the influence of local growth factors and are, unlike basophils, usually not found in the circulation after maturation. MCs are found throughout the body, primarily in tissues that come in contact with the outside of the body for example mucus membranes in the digestive system and the respiratory tract. MCs are important cells in various inflammatory conditions by releasing the contents of their secretory granules which promotes inflammation and in combating infections caused by extracellular parasites. The contents of the granules include several preformed inflammatory mediators such as histamine, cytokines, serglycin proteoglycans and proteases of three different classes. The MC proteases are tryptases, chymases and carboxypetidase A (CPA). In addition to the preformed substances released there is also *de novo* synthesis of arachidonic acid metabolites such as leukotrienes-C_4 and prostaglandin-D_2 by activated mast cells (Hallgren *et al* 2006, Hallgren *et al* 2004). Human MCs are divided into two populations, mucosal mast cells (MMCs) primarily found in the mucosa of the gastrointestinal tract and the airways and connective tissue mast cells (CTMCs) primarily found in the skin and serosal cavity. MCs can also be classified according to the contents of the granules. MC_T contain tryptase while MC_{TC} contain tryptase as well as chymase and CPA. Activation of MCs can occur as part of the adaptive immune response when an antigen binds to two IgE antibodies that bind to specific receptors, FcεRI, on the mast cell surface. The IgE antibodies are produced by plasma cells as a response to encountering an antigen such as pollen or a parasite and bind with high affinity to the FcεRI. This cross-linking of IgE molecules stimulates degranulation with release of preformed inflammatory mediators and *de novo* synthesis of others (see above). MCs can also be activated directly via receptors on its cell surface by for instance complement factors such as C5a and C3a, different drugs, different pathogens and direct cell-cell interactions with activated T cells (Roitt *et al* 2001).

Tryptase and proteoglycans

Tryptase is a protease (a proteolytic enzyme) named for its preference to cleave substrates in a trypsin-like way at the C-terminal end of the amino acid residues of Lysine (Lys) and Arginine (Arg), a specificity also seen in the pancreatic enzyme trypsin. Several different tryptases have been found in MCs in different species (Hallgren *et al* 2006). In humans several MC tryptases are known, they include α-, β-, γ-, δ- tryptase and transmembrane tryptase (TMT) (Jogie-Brahim *et al* 2004) with β-tryptase being the main form stored in human MCs. β-tryptase is further divided into three isoenzymes, βI, βII and βIII. βI and βII differ in only one amino acid whereas βIII differ in an additional three amino acids compared to βI/βII. βI and βII differ at position 102 where βI has Asn and βII has Lys. Asn 102 is part of an N-glycosylation site and βI is glycosylated at this position while β-II is unglycosylated (Vanderslice *et al* 1990).

Tryptase is stored in MC granules in its active form as a tetramer in a complex with heparin proteoglycan (heparin PG) or chondroitin sulphate proteoglycan (CSPG). In the tetramer the active sites of the tryptase molecules are facing a central pore, a structure that limits the number of substrates that can be cleaved due to the size of the substrates. This tetramer structure also makes it difficult for tryptase inhibitors to be effective. There are two types of proteoglycans expressed by human MCs, heparin PG and CSPG. Heparin is a glycosaminoglycan (GAG) consisting of repeating disaccharide units of glucuronic acid or iduronic acid and glucosamine. Heparin is negatively charged due to a high number of sulphate groups. Heparin PG is made up of a protein core (serglycin) to which the GAGs are attached. It has been shown that heparin PG, or other negatively charged polysaccharides, is required to form active tryptase tetramers of mouse mast cell protease 6 (mMCP-6) and that formation of tetramers occur at acidic pH (Hallgren *et al* 2000). Chondroitin sulphate proteoglycan has the same type of protein core as heparin PG while the GAGs are made up from repeating disaccharide units of glucuronic acid and galactosamine. CSPG is normally not as negatively charged as heparin PG.

The aim of this study has been to investigate the role of heparin in the activation and formation of active tetramers of human βI-tryptase and βII-tryptase.

MATERIALS AND METHODS

Recombinant βI/ βII-tryptase DNA had previously been ligated into a pCEP Pu2 vector, transformed into chemo-competent *E.* coli (MC1061) and after confirming the correct DNA sequence transfected into 293-EBNA cells (Hallgren *et al* 2004).

Protein expression

Recombinant β-tryptase was expressed in 293-EBNA cells in serum-free DMEM (National Veterinary Institute, Uppsala) supplemented with 2mM L-glutamine (National Veterinary Institute, Uppsala), 50μg/ml Gentamycin (National Veterinary Institute, Uppsala), 5 μg/ml Puromycin (Sigma, Steinheim, Germany) and 10% FCS (Invitrogen, Life Technologies, Groningen, The Netherlands) at 37ºC and 5% CO_2. Puromycin was used for selection of cells containing the pCEP Pu2 vector and Gentamycin was used as a general antibiotic.

Purification of recombinant tryptase

The recombinant β-tryptase construct contained a histidine-tag (6 x His) and an enterokinase (EK) cleavage site (Asp-Asp-Asp-Asp-Lys) between the His-tag and the mature β-tryptase sequence. Recombinant tryptase protein was purified from conditioned media collected from the transfected 293-EBNA cells. Conditioned media was collected and centrifuged at 418 x g for 10 min, the supernatants were collected and stored at- 20º C while awaiting purification. Ni-NTA agarose 0.5 ml (QIAGEN, GmbH, Hilden, Germany) was loaded on a 10 ml Poly-prep Chromatoghraphy column (Bio-Rad, Hercules, USA) and washed with dH_2O. Thawed media (~750 ml) was applied to the column. The column was then washed with 3 x 10ml PBS (pH 7.5) + 0,1% Tween containing 1M-2M NaCl and the protein eluted with 100mM Imidazol in PBS pH 7.5. The fractions collected were analyzed by SDS-PAGE in a Laemmli system and stained with Coomassie Brilliant Blue. Fractions with pure tryptase were pooled and stored at 4ºC. Concentration of tryptase was determined by measuring A_{280} (Lambert Beers law) using a calculated molar extinction coefficient of 64 970 M^{-1} cm^{-1}.

Enterokinase cleavage

To remove the 6 x His-tag and EK cleavage peptide the fusion protein was incubated with enterokinase (Roche Molecular Biochemicals, Mannheim, Germany) at an EK/fusion protein ratio of 1:500 in PBS pH 6.0 at 37 ºC for 15 hours.

Enzymatic assays

Tryptase activity was measured in 96-well microtiter plates. 250 ng of EK-digested fusion protein was incubated in PBS with pH ranging from 5.5-7.5, either in the presence or absence of heparin and in a total volume of 130-200 μl, for 30 min at room temperature. Thereafter 20 μl of S-2288, a chromogenic substrate (2mM; H-D-Ile-Pro-Arg-pNA; Chromogenix, Mölndal, Sweden), was added and the absorbance at 405 nm was recorded with a spectrophotometer (Titertek Multiscan, Flow Laboratories). In another experiment 250 ng of tryptase was incubated in PBS pH 6.0 with heparin in concentrations ranging from 0-1000μg/250ng tryptase for 30 min at room temperature before addition of S-2288 and measurement of activity was performed as described previously. Activities

were determined as the average of three measurements and the results are given with ± standard deviation.

Heparin-Sepharose chromatography

Affinity chromatography was performed on a 10 ml Poly-Prep Chromatography column containing approximately 0.5 ml heparin-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was equilibrated with PBS pH 6.0 or 7.5. EK-digested β-tryptase, 40 µg in a total volume of 320 µl, was incubated for 15 min on the column. The protein was then eluted by washing the column with 400 µl of PBS (pH 6.0 or 7.5) with an increasing concentration of NaCl (ranging from 0.15M to 2.0M). Samples from each fraction (10 µl) were incubated with PBS pH 6.0 (105 µl) and 1 µg heparin (10 µl of 0,1 µg/µl) at room temperature for 30 min. Tryptase activity was measured after addition of S-2288 as described above.

RESULTS AND DISCUSSION

Expression of human βI-and βII-tryptase

Human βI-and βII-tryptases were expressed in 293-EBNA cells, a human cell line system. Recombinant β-tryptase has previously been expressed in insect cells (Huang *et al 2001*, Wang *et al* 1998, Sakai *et al* 1996) or in yeast (Chan *et al* 1999, Niles *et al* 1998) but to our knowledge this is the first report describing expression in a human system. The recombinant fusion proteins were purified on Ni-NTA agarose and cleaved by EK-digestion, obtaining the mature forms of βI- and βII-tryptase monomers (Fig. 1)

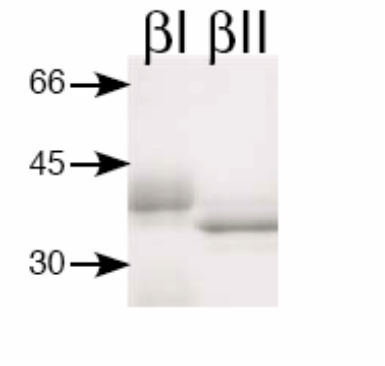


Fig. 1*Recombinant βI-tryptase and βI-tryptase purified from cell culture medium, digested with EK and analyzed on SDS-PAGE.*

The βI-tryptase is showing a larger size and a somewhat fuzzy appearance compared to βII-tryptase on SDS-PAGE. This indicates that the βI-tryptase is glycosylated at Asn 102. The glycosylation of βI-tryptase is the only major difference in the two isoforms since they only differ in one amino acid, 102.

Activation of human βI-and βII-tryptase

Human βI-and βII-tryptase fusion proteins were digested with EK at pH ranging from 5.5-7.5 and then incubated with or without heparin. After incubation the sample was taken to pH7.5 to obtain optimal conditions for substrate cleavage and the chromogenic substrate S-2288 was added. The enzymatic activity of the tryptase was then measured. In the absence of heparin tryptase activity was low or non-detectable for both βI-and βII-tryptase over the investigated pH-range. In the presence of heparin however, enzymatic activity was readily detected in both βI- and βII-tryptase, with optimal activity of βI-tryptase incubated with heparin in low pH (5.5-6.0) and a marked decline in activity when reaching neutral pH. This in contrast to βII-tryptase which showed only a moderate pH-dependence (Fig. 2).

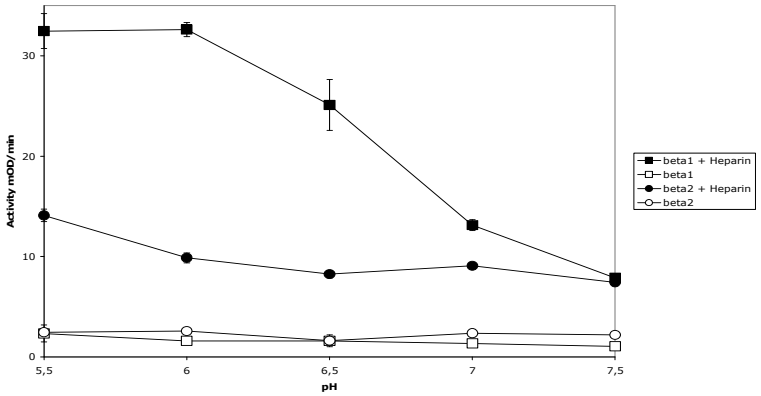
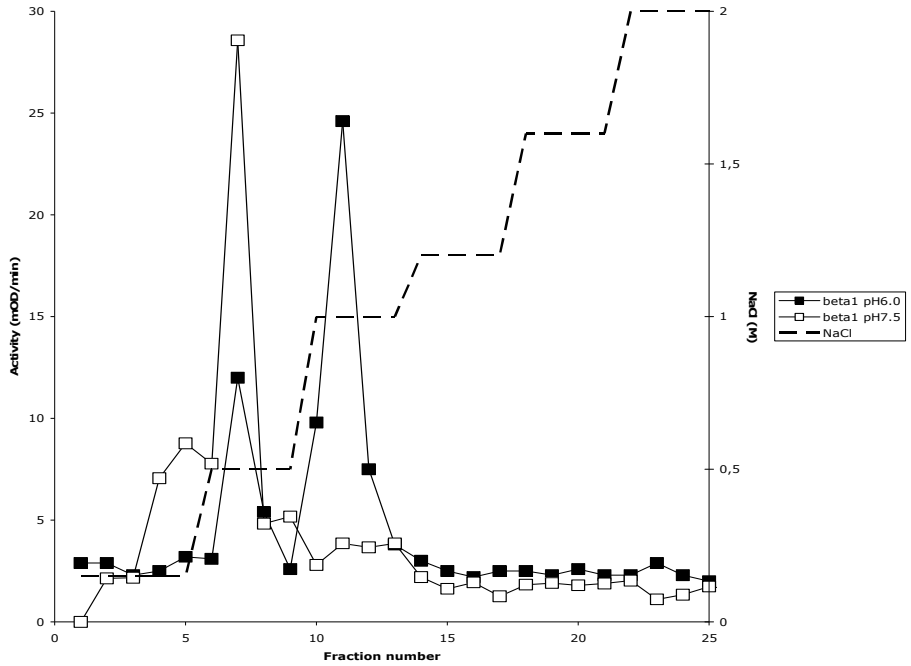


Fig. 2 *Enzyme activity of βI-tryptase and βII-tryptase incubated with heparin at pH 5.5-7.5.*

These results indicate that human βI-and βII-tryptase interact with heparin to become active and that βI-tryptase reaches optimal activity when this interaction occurs at acidic pH, whereas βII-tryptase appears to be less pH-dependent. To further investigate this hypothesis of pH-dependence βI-and βII-tryptase were analyzed by affinity chromatography on a heparin-Sepharose column equilibrated with PBS pH 6.0 or 7.5. The tryptase was eluted from the column step-wise with NaCl in increasing concentrations (0.15M-2M). Both βI-and βII-tryptase bound strongly to the column at pH 6.0 where approximately 1M NaCl was required for elution, a result indicating high affinity for heparin. At pH 7.5 it appears that both βI-and βII-tryptase have a considerably lower affinity for heparin since the major portion of the sample protein eluted at 0.5M NaCl or less (Fig. 3).

A



B

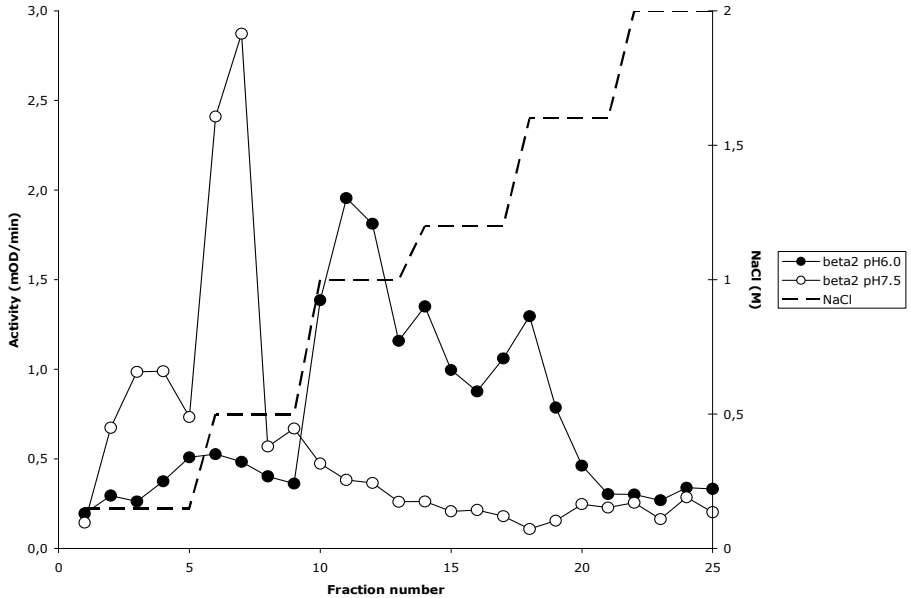


Fig. 3 *Affinity chromatography of βI-tryptase (A) and βII-tryptase (B) on heparin-Sepharose at pH 6.0 or 7.5.*

Dose response experiments where βI-and βII-tryptase were incubated with different concentrations of heparin (0.007-7000 μg/ml) showed enzyme activity optimums at ~7 μg/ml of heparin for both βI-and βII-tryptase. βI-tryptase generally showed substantially higher activity levels than βII-tryptase. At heparin concentrations above 7 μg/ml enzyme activity levels decreased for both βI-and βII-tryptase. At the highest concentrations of heparin in the experiment enzyme activity levels approached those obtained in absence of heparin. βI-and βII-tryptase show a bell-shaped dose response curve (Fig. 4).

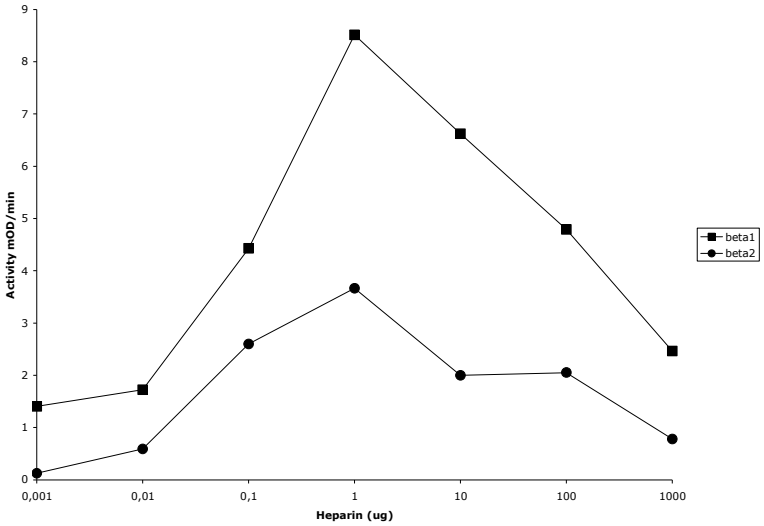


Fig. 4 *Dose response for heparin dependent activation of βI-tryptase and βII-tryptase.*

Discussion

Tryptase is one of the main inflammatory mediators released by mast cell degranulation. Tryptase is stored with heparin proteoglycan in its active form in the granules. It has previously been shown that recombinant human β-tryptase, expressed in insect cells, required heparin to become active (Huang *et al* 2001). In this study recombinant human β-tryptase was expressed in a human cell line, 293-EBNA cells, to ensure correct folding and glycosylation of the tryptase. We have shown in this study that both βI-tryptase and βII-tryptase are dependent on heparin to exhibit enzymatic activity. In a parallel study it was shown that heparin was also needed for tetramerization of β-tryptase (Hallgren *et al* 2004).

We show that the activation of both βI-tryptase and βII-tryptase is strongly dependent on heparin and we also demonstrate that the heparin-induced activation shows a pH-dependence with activation being optimal at slightly acidic pH (5.5-6.0). However, human β-tryptase was also activated at neutral pH which differs from the results from a previous study on mMCP-6 showing barely detectable activity at neutral pH (Hallgren *et al* 2000). Human βII-tryptase showed a lower

pH-dependence for activation than βI-tryptase and the reason for this is not clear. The structural difference between βI-tryptase and βII-tryptase is at amino acid 102 where βI-tryptase has a glycosylation site (Asn102) and βII-tryptase instead has a Lys residue and is not glycosylated at that site. The difference in function between the isoenzymes is therefore suggested to be related to the difference in glycosylation, possibly that the oligosaccharide on βI-tryptase facilitates binding to heparin. However both βI-tryptase and βII-tryptase show approximately similar affinity for heparin in the experiment using heparin-Sepharose for affinity chromatography leading to the hypothesis that the oligosaccharide on βI-tryptase is important for assembly of active tetramers without affecting affinity for heparin. The parallel study on the subject indeed revealed that βI-tryptase primarily formed active tetramers in presence of heparin while βII-tryptase in the presence of heparin predominantly formed inactive complexes of larger size than tetramer (Hallgren *et al* 2004), something that could account for the generally lower activity level of βII-tryptase.

The activation mechanism for human β-tryptase is similar to that suggested for mMCP-6 (Hallgren *et al* 2001) with tryptase monomers binding heparin proteoglycan in dimers and then assembling dimers into tetramers. Activation of human β-tryptase by heparin show a bell shaped dose response curve, an indication of tri-molecular interaction and therefore compatible with tryptase monomers being cross linked by heparin. The parallel study also showed that β-tryptase appear to have the same requirements regarding the size of heparin oligosaccharide required for binding monomers as mMCP-6 does and that β-tryptase also can occur as active monomers given the appropriate conditions (Hallgren *et al* 2004).

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