HIGH-YIELD RECOMBINANT EXPRESSION OF THE EXTREMOPHILE ENZYME, BEE HYALURONIDASE IN PICHIA PASTORIS

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Running title: Enzymatic characterization of recombinant bee hyaluronidase

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Abstract

Hyaluronidase from honey bee was recombinantly expressed as a secreted glycoprotein in *Pichia pastoris*. The active enzyme was produced in milligram quantities per liter of primary culture. When changing the codons of the original transcript to triplet sequences preferred by *P. pastoris*, no further increase of protein product could be achieved. After expression of a fusion protein by linking hyaluronidase and human serum albumin together with the recognition sequence for the protease, factorXa, fragmented protein products were obtained in the culture supernatant. Only after replacement of the hinge region with a serine-glycine-rich linker, stable full-length fusion protein could be generated. The protein products were purified by cation exchange chromatography at pH 5.0 and pure enzyme fractions were further characterized in detail. The biochemical properties of the product matched those of crude hyaluronidase within bee venom: the native and the recombinant enzyme exhibited activity over a pH range from 3 to 8 (maximum: 3.8), at temperatures as low as 4°C and up to 90°C (maximum 62°C), and at ionic strength as high as 2 M salt. Recombinant bee hyaluronidase efficiently degrades 6-*S*-chondroitin sulfate (chondroitin sulfate C) as well as 4-*S*-chondroitin sulfate (chondroitin sulfate A), the latter to a lesser extent. Only very little hydrolase activity towards chondroitin sulfate B (dermatan sulfate) was detectable.

(212 words)

Introduction

Hyaluronidases are enzymes that degrade extracellular glycosidic coats [1] commonly known as „ground substance“ which is made up to a large extent of hyaluronan (HA).
Hyaluronidases have been found in prokaryotes as well as in eukaryotes [2]. Hyaluronidases have different catalytic mechanism, substrate specificities, affinities to inhibitors, and notably, different pH optima. Mammalian-type hyaluronidases have been grouped in EC 3.2.1.35 [3]. This group consists of endo-ß-*N*-acetyl-hexos-aminidases that cleave the ß-1,4 glycosidic linkage between *N*-acetyl-glucosamine and glucuronate within the HA chain, producing non-reactive oligomere end products. In various organisms, venoms such as those of bees, wasps, spiders, scorpions, fish, snakes and lizards, hyaluronidases enhance the noxious action of substances by helping to spread constituents of the venom cocktail at the injection site [4]. Notably, the first structure of mammalian-type hyaluronidase could be deduced from a cDNA clone from venom glands of honey bees [5]. So far, the three-dimensional structure of hyaluronidases from bee and wasp have been deciphered in detail [6, 7].
PH-20 prepared from bovine or ovine testes has been applied in the clinics in order to render tissues more susceptible for drugs [8]. Supplementation of specific medications with bull or ram sperm-derived hyaluronidase is already used when applying chemotherapeutics, anesthetics and analgesics. Furthermore, this hyaluronidase is also used in ophthalmologic surgery [9], in the treatment of diseases associated with excess “ground substance” in the extracellular space to enhance circulation of physiological fluids at the site of administration (e.g., as a spreading agent) [10], and is applied subcutaneously or in a topical fashion [11]. With some success, hyaluronidases have been used in anti-cancer regimens. Tumors that appear to be resistant to chemotherapy, can be rendered sensitive when the mammalian sperm-derived PH-20-type hyaluronidase is added [12-16]. Unfortunately, cases have been reported where patient’s immune system have not tolerated treatment with PH-20 hyaluronidase [17, 18]. In line with this, it appears most desirable to develop strategies to produce hyaluronidase as a recombinant enzyme, which is superior to products that are routinely used in the clinics and conclusively could be of commercial relevance.

In this context we reasoned whether bee hyaluronidase actually matches the mentioned criteria much better than its known mammalian relatives. However, bee hyaluronidase has not been subject to a thorough biochemical characterization, mainly because of the difficulty to purify sufficient amounts of the enzyme from venom for further studies [19]. The structure of

hymenoptera enzymes is compact comprising only of one domain; in other words, it lacks the C-terminal part found in all mammalian HYAL proteins. Moreover, the biological function of the bee enzyme appeared to have been specifically evolved to enhance spreading of venom after a sting and this type of enzyme therefore should actually exert properties necessary to efficiently hydrolyze HA and related glycosaminoglycans in compact tissues or body fluids. Working along this line, we decided to produce larger amounts of bee hyaluronidase by performing heterologous expression in *Pichia pastoris,* which after purification would permit a thorough biochemical characterization of the recombinant protein product.

Material and Methods

Cloning and construction of yeast expression vectors - The cDNA encoding the entire open reading frame of bee hyaluronidase was kindly provided by Günther Kreil, Austrian Academy of Sciences, Salzburg, Austria (accession number L10710). A codon-modified cDNA of bee hyaluronidase ($Hya^{\Delta C}$) was generated by *de novo* gene synthesis (GenScript Corporation, Piscataway, NJ, USA). The protein-coding regions of both genes were amplified by PCR using the following primers: for Hya: 5'-AAAGAATTCACCCCCGACAACAACAAAACCGTACGG-3' and 5'-TCTTGCGGCCGCTCACACTTGGTCCACGCTCACGTC-3'; for $Hya^{\Delta C}$: 5'-GAGGAATTCACTCCAGATAACAATAAGACTGTT -3' and 5'-CTCTGCGGCCGCTTAAACTTGATCAACAGAAAC - 3' (MWG, Germany). Amplified cDNA lacking the information for the original leader peptides was cloned between the *Eco*R I and *Not* I sites of the yeast expression vector pPIC9 (Invitrogen, accession number: Z46233) in frame with the α-factor signaling sequence. The cDNA of human serum albumin (accession number NM_000477; gift from A. Bito, University of Salzburg, Austria) without the signal peptide was cloned between *Eco*R I and *Sna*B I sites of pPIC9-$Hya^{\Delta C}$ and the albumin-coding region and $Hya^{\Delta C}$ were merged with the following DNA sequence; for construct F-x: 5'-ATCGAAGGTCGTACGCGT -3' and for F-l: 5'- GGTGGATCTGGAGGT -3'. Unless otherwise stated, all plasmids were linearized with *Sal* I prior to transformation into *Pichia* cells.

Recombinant protein expression - The coding region of bee hyaluronidase was cloned into bacterial expression vector pMW172 [5] under the control of bacteriophage T7 promoter (gift from G. Kreil). Recombinant protein expression was achieved in *E.coli* BL21 DE3 cells by addition of isopropyl β-D-1-thiogalactopyranoside (Sigma) to a concentration of 0.1 mM for 4 hours after bacterial cells have been grown at 37°C to an optical density of OD_{600} ~ 0.9 in LB-media (10 g/L peptone, 5 g/L yeast extract, 5 g/L sodium chloride, pH 7.5) containing 120 mg/mL ampicillin. Total protein of harvested cells was separated on a 12% polyacrylamide gel using Tris-glycine-SDS (TGS) buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). The protein band containing recombinant bee hyaluronidase (about 41 kDa) was excised, the gel fragment was transferred to a dialysis tubing (cut-off 10 kDa), and the protein was eluted from the gel by electrophoretic means in TGS buffer. The resulting protein solution was

centrifuged to remove insoluble material and then dialyzed against phosphate-buffered saline pH 7.4. Before immunization, aliquots of 0.5 mL were frozen at -20°C until use.
For expression in the *Pichia pastoris* strain GS115 (*his4*), pPIC9-Hya was transformed by electroporation as described previously [20]. *Pichia* recombinant for bee hyaluronidase was used to inoculate 50 mL of YPD medium. A 20 mL overnight culture was transferred to 2 L YPD and incubated in two 2 L baffled Erlenmeyer flasks rocking at 200 rpm at 30°C. After 3 days when the yeast culture had reached an OD_{600nm} of 12-18, cells were collected by centrifugation for 15 minutes at 1,600 x g and 20°C (Beckman Coulter, JA-10 rotor). The cell pellet derived from 1 L was resuspended in 100 mL buffered methanol minimal medium (BMM, 100 mM potassium phosphate, 0.34% yeast nitrogen base without amino acids, 4 x 10^{-5}% biotin, 0.5% ammonium sulfate) containing 1% methanol in an 1 L baffled Erlenmeyer flask. Yeast cells were incubated for 3 days and methanol (1% v/v) was added daily. Then cells were removed by centrifugation for 15 minutes at 1,600 x g and 4°C and supernatant was transferred to new tubes and centrifuged again for 30 minutes at 3,200 x g and 4°C to remove cell debris. Protein containing supernatant was filtrated through a 0.45 µm syringe filter and stored at -20°C for further use.

Ion exchange chromatography - Pichia supernatant was diluted with 3 volumes of 50 mM sodium acetate pH 5.0 and applied (1 mL/min) onto a cation exchange column (1 mL Hitrap HP SP, GE Healthcare) in a cold room. After loading, the column was washed with 10 mL 50 mM sodium acetate pH 5.0. Hyaluronidase was eluted applying a salt gradient 0 – 1 M NaCl. Pooled fractions were subsequently desalted by Sephadex G50 chromatography (GE Healthcare) and the resulting protein was lyophilized for long-term storage.

Polyclonal antibody production - Chinchilla mixed breed rabbits were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed in temperature-and humidity-controlled rooms with a 12- hour light/dark cycle at the Central Laboratory Animal Facilities, Innsbruck Medical University. Rabbits were fed standard chow-diet and received tap water *ad libitum.* Permission for animal experimentation was granted (MBBWK GZ 66.015/13-BrGt/2002).
Prior to each immunization, blood samples (4 mL) were drawn from the marginal vein of the rabbit ear, centrifuged, and sera were stored for later determination of antibody titer by ELISA. For the first immunization 200 µg of antigen was mixed with 400 µL of Freund's complete adjuvant (ICN). For subsequent immunizations 500 µg of antigen was mixed with

an equal volume of Freund's incomplete adjuvant (ICN). Subcutaneous injections were performed on day 1, 30, 57, and 98 followed by total blood collection on day 112. Plasma was isolated from blood samples containing heparin (5 IU/mL) by centrifugation at 500 x *g* for 20 min (Beckman CS-6R). One mL aliquots were frozen at -70° C for later use.

Protein electrophoresis, Western analysis - Protein samples were separated on 10% polyacrylamide gel, blotted onto a PVDF membrane (BioRad). Hyaluronidase was detected using primary antibody at a dilution of 1:2500, secondary horse radish peroxidase anti-rabbit IgG conjugates (1:5000, Pierce), enhanced chemiluminescence (Pierce) and a fluorescence imager (FluorChem HD2, Alpha Innotech)

Enzymatic activity - Degradation of glycosaminoglycans was measured by quantification of free reducing ends of hydrolyzed substrate [21] using *N*-acetylglucosamine as a standard. In a total volume of 100 µL, reactions contained recombinant hyaluronidase, or bee venom (lot 01181, Heinrich Mack Nachf., Illertissen, Bavaria), 500 µg/mL substrate, buffer and varying salt concentrations as indicated. The mixture was prepared on ice and incubated at 37°C for 20 minutes. The reaction was stopped by heating for 5 minutes at 95°C. Next, 20% (v/v) of an 800 mM $K_2B_4O_7x7H_2O$ solution was added and the sample was again heated to 95°C for 3 minutes. After chilling on ice, 5 volumes of acetic acid containing 1% *p*-di-methyl-amino-benzaldehyde, and 1% HCl (37% w/v) were added and the sample was incubated for 20 minutes at 37°C. The resulting optical density was measured at 585 nm. One unit of hyaluronidase activity was defined as of corresponding to the liberation of 100 nmol of reducing ends in 20 minutes at 37°C in the presence of 0.5 mg/mL HA.

Results & Discussion

We first expressed hyaluronidase in *E.coli* and successfully refolded insoluble material to yield active enzyme (data not shown) as previously reported by others [22]. These preparations were injected into rabbit to raise polyclonal antibodies, which during the course of the subsequent work, presented in this contribution, were employed to detect bee hyaluronidase by Western blotting in the subsequent analyses. Unfortunately, we were unable to purify these preparations to homogeneity. We therefore expressed the protein recombinantly in *Pichia pastoris* GS115. By now, a number of industrially as well as pharmaceutically interesting proteins have been manufactured using this particular expression system, e.g. human serum albumin (HSA), lysozyme or collagen [23-25]. Compared to other expression systems, *Pichia* offers many advantages: due to the poor fermenting activities, cells can be grown to high density, which is considered the major advantage relative to *S.cerevisiae* [26]; moreover the cell culture supernatant is essentially endotoxin-free, and secretory molecules are efficiently secreted into the culture medium, which only contains trace amounts of other proteins, a fact that eventually also greatly eases subsequent purification. Lastly, these protein preparations are not contaminated with viruses, which could be a problem, when producing biomolecules in animal cell culture.

Employing the expression plasmid pPIC9 together with the signal sequence of α-factor of *S.cerevisiae*, active hyaluronidase in the range of >10 mg/mL were secreted into the culture medium. More enzyme was produced when the vector was restricted with *Sal* I instead of *Sac* I prior to recombination (Fig. 1). The secreted recombinant enzyme could be efficiently separated from other ingredients present in the culture medium by ion exchange chromatography (S-Sepharose at pH 5.0). Elution at approximately 250 mM NaCl yielded a concentrated preparation (Fig. 2) (Table 1). The enriched fractions were desalted (Sephadex in water) and subsequently lyophilized. The isolated protein was further specified by N-terminal protein sequencing applying automated Edman degradation. 60% started at Phe-(-1), 24% at Tyr-(-4) and 16% at Thr-(+1) with regard to the predicted N-terminus of bee hyaluronidase (Fig. 3), indicative for post-translational processing taking place during passage through the secretory pathway. No further proteins were detectable applying this technique. Following this procedure, more than 10 mg of active enzyme are regularly being obtained from one liter of primary culture.

Grantham et al. proposed that organisms display a non-random pattern of synonymous codons [27, 28]. Later on, whole-genome analyses of coding sequences substantiated the notion that codon usage may correlate with the level of gene expression [29]. After validating the bee hyaluronidase cDNA with regard to *P.pastoris* codon usage frequency, we noted that bee hyaluronidase cDNA contains many codons, which are rarely used in *Pichia* and thus the original sequence may be a suboptimal template for heterologous protein production in this yeast strain. As previously shown, change of coding regions towards the codon bias of the host cell can yield an average 10- to 50-fold increase in a variety of host cells [30-33]. This body of evidence suggested to us that changing codons within the hyaluronidase cDNA towards the bias of *P. pastoris* could have a positive impact on expression levels. Using the information of preferred *P. pastoris* codons as provided by Genscript, Inc., the native sequence was *de novo* synthesized to create a fully codon-optimized cDNA ($Hya^{\Delta c}$) and the synthetic gene was expressed in *Pichia*. The respective sequence information was deposited in Genbank (accession number EU152302). For three pairs of individual yeast clones, the amount of secreted hyaluronidase was further examined by Western blot (Fig. 4A), the protein content was monitored during purification by UV spectroscopy (Fig. 4B), the resulting enzymatic activity was assayed (Fig. 4C), and the copy number of the plasmids inserted into the *Pichia* genome was determined. However, Hya^{wt} and $Hya^{\Delta C}$ exhibited equivalent expression levels. In view of these data, we surmised that due to the tight regulation required for effective extracellular targeting and trafficking through the secretory pathway, which also includes glycosylation of hyaluronidase, an increase of protein yield may only be achieved by further single or combinatorial mutations within individual codons.

When expressing HSA under the control of the AOX1 promoter, we experienced production of secreted HSA at amounts much higher than those found with hyaluronidase (data not shown). We reasoned whether combining cDNAs of HSA and hyaluronidase in a way that a fusion protein consisting of HSA together with hyaluronidase on its C-terminus could also be expressed after recombination into the *P.pastoris* genome. It is generally accepted that serum albumin fusion proteins are suitable for biopharmaceutical applications [34-37]. Potential protein therapeutics that have been modified in the manufacturing process, include various protein conjugates; e.g., serum albumin has been coupled to a glucagon-like peptide 1 [38], or antithrombic agents [39, 40] to improve circulatory half-life. We therefore constructed two variants composed of HSA and hyaluronidase merged through two different linker sequences. The first construct included the recognition sequence for factorXa protease (F-x), the other

sequence was composed mainly of glycine and serine residues (F-l) (Fig. 5A). Interestingly, only F-l was expressed as a full length protein, whereas in case of F-x only trace amounts of complete fusion protein could be detected by Western analysis (Fig. 5B). Expression of F-x contained proteins comprising molecular weights of native HSA and bee hyaluronidase. Comparable results have previously been evidenced by Daly and Hearn, 2006 [41], who also attempted to express fusion protein constructs that include factorXa recognition site (IEGR). They reported the presence of two proteases kex2p and ste13 being present at significant levels in *P.pastoris.* Furthermore, kex2p appeared to exhibit activity albeit at reduced rates towards factorXa recognition sites. It appears likely that kex2p activity is however high enough for the cleavage of proteins that are traveling through the secretory pathway and are exposing a IEGR-containing sequence stretch. In contrast to what we experienced with F-x, F-l was secreted at its expected full-length and furthermore was found to be enzymatically active at rates comparable to native hyaluronidase. In addition to that, the overall yield of the F-l fusion protein was approximately the same as that obtained when expressing hyaluronidase alone.

After purification, we characterized the recombinant enzyme side-by-side with its native counterpart as contained in complete bee venom by assessing the activity at varying pH, temperature and salt concentrations. When comparing these results (Fig. 6), only subtle differences between the two preparations could be observed, which we refer to the complex mixture of crude venom that has been used for this comparative investigation. We therefore concluded that active bee hyaluronidase can be efficiently produced in *P.pastoris* as an active enzyme, which is indistinguishable from the native enzyme. We furthermore uncovered native bee hyaluronidase and the recombinantly expressed protein as being remarkably robust enzymes remaining active at extreme conditions such as pH lower than 4 and high ionic strength buffers containing more than 1 M of salt. In particular, the enzyme displayed significant levels of activities at temperatures as low as 4°C (34%) and still exhibited hydrolase activity at 90°C to an extent of 19% in comparison to the optimum.

We furthermore monitored substrate specificities of purified recombinant bee hyaluronidase towards other glycosaminoglycans (Fig. 7) and revealed hydrolytic activity against chondroitin sulfate A (CS-A) and chondroitin sulfate C (CS-C). Notably, the enzyme is capable of degrading CS-C at a rate of around 40% compared to its hyaluronidase activity. Only trace amounts of chondroitin sulfate B (CS-B/DS), also called dermatan sulfate were

degradable. This is in line with the enzymatic properties of other venom hyaluronidases, which are readily hydrolyzing CS-A and CS-C, yet exhibit only little affinity towards dermatan sulfate as a substrate [42]. It is well documented that depending on the tissue sources, from which CS has been isolated, the molecular composition of this class of glycosaminoglycans greatly varies [43]. For instance, the ratio of 4- to 6- sulfation of CS chains is significantly changed with tumor progression [44], in atherosclerotic lesion development [45] and during aging of various organisms [46, 47]. In light of these results, it seems deceptive that this enzyme is called nothing more than hyaluronidase. CS-A and CS-C may be either efficiently or partially degraded by bee hyaluronidases or related enzymes while due to its different molecular structure containing iduronic acid instead of glucuronic acid, CS-B/DS, is by and large neglected as a substrate. Hence, applications either employing bee hyaluronidase for biochemical or medical purposes are now conceivable.

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Table 1: Purification of recombinant bee hyaluronidase.

Step	Volume (mL)	Activity (U/mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purifi-cation factor
Culture supernatant	10	1.01	10.12	20.51	0.49	100	1
SP ion exchange	6	0.91	5.50	1.17	4.77	54.8	9.6

Figure legends

Fig. 1. Expression vector pPIC9 bearing the cDNA of bee hyaluronidase was either linearized with *Sac* I or *Sal* I prior transformation. *Pichia pastoris* cells were cultured in buffered-minimal methanol medium for protein production for > 3 days and 50 µL aliquots were further analysed by SDS PAGE (A) and Western blot (B). Filled circles specify recombinant hyaluronidase at ~ 50 kDa. The lower band appeared to be specific for *Pichia* (arrow).

Fig. 2. Purification of recombinant bee hyaluronidase (Hya) from *P.pastoris* supernatant. (A) Cation-exchange chromatography of 10 mL recombinant *Pichia* derived supernatant. Collected fractions (1.8 mL) are indicated at the bottom. Elution of protein as monitored by absorption at 280 nm (continuous line in mAu, left) was achieved through increasing ionic strength with NaCl as indicated (dashed line in mS/cm, right). (B) Enzymatic activity was assessed for each fraction in triplicates. (C) Coomassie stained SDS-PAGE of native GS115 *P.pastoris* supernatant (mock), transformed yeast supernatant (H), H after sterile filtration using a 0.22 µm syringe filter (H_{SF}), H_{SF} diluted with loading buffer (pH 5.0) one to four (H_{SF}1:4), the column flow through (FT), and the collected wash after loading, fraction 7, 8 and 14 of the elution by means of increasing ionic strength up to 1 M NaCl (F7, F8, F14).

Fig. 3. Pre-pro-sequence of hyaluronidase comprising of α-factor signal peptide and the N-terminus of native hyaluronidase. Processing sites as determined by Edman degradation are indicated.

Fig. 4. Comparison of bee hyaluronidase constructs with native codons (Hya^{wt}) versus a codon-modified construct ($Hya^{\Delta C}$). (A) Hyaluronidase production of three individual wild type and codon-variant *Pichia* clones, named Hya^{wt} and $Hya^{\Delta C}$ was compared by means of Western detection of culture medium. Clone 3 contained twice the relative copy number of hyaluronidase gene when compared to clone 1 and 2 by means of quantitative PCR (B) The expressed protein levels of all three Hya^{wt} and $Hya^{\Delta C}$ were determined after elution of 2 mL *Pichia* supernatant from a cation-exchange column performing UV spectrogram peak integration and (C) quantitative assessment of hyaluronidase activity.

Fig. 5. Fusion proteins comprising of human serum albumin (HSA) together with hyaluronidase (Hya). (A) Illustration of two variants, F-x and F-l, depicting HSA and Hya with their respective N and C-terminal amino acid moieties together with central hinge

regions. (B) Expression of either Hya (filled square) or HSA (filled circle) alone as well as fusion variants (asterix) were monitored by SDS-PAGE followed by Coomassie Blue staining (left panel) and Western detection (right panel). Molecular weight in kDa is indicated between the two images.

Fig. 6. Enzymatic characterization of recombinant bee hyaluronidase (120 ng) (black line) compared to hyaluronidase-containing bee venom (75 µg)(grey dashed line) in a 100 µL reaction. Hyaluronidase activity was assessed in triplicates at varying pH at 37°C (A), varying incubation temperatures (B) and NaCl concentrations as indicated in mM (at pH 5.0 and 37°C) (C).

Fig. 7. Enzymatic activity of recombinant bee hyaluronidase at standard conditions of 100 mM sodium phosphate, pH 5.0 at 37°C applying chondroitin sulfate A, B and C as substrates. The rate of the respective specific activity was assessed in triplicates and referred to the respective hyaluronidase activity.

Figure 1

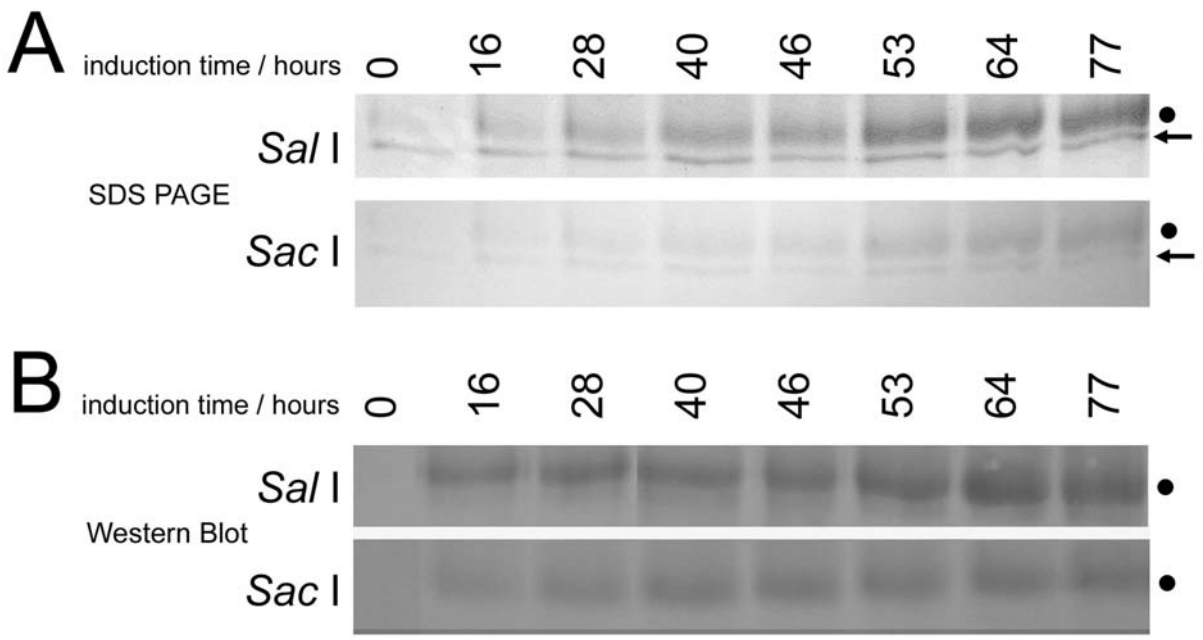
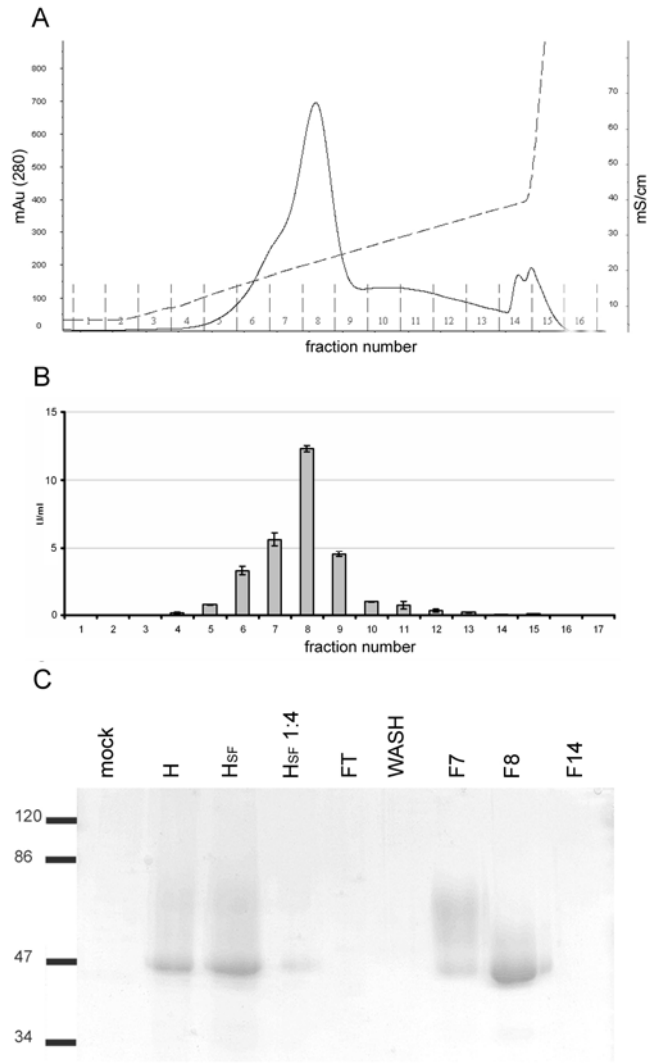

A
induction time / hours
0
16
28
40
46
53
64
77
Sal I
SDS PAGE
Sac I
B
induction time / hours
0
16
28
40
46
53
64
77
Sal I
Western Blot
Sac I

Figure 2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mAu (280)
mS/cm
fraction number
B
fraction number
C
mock
H
HSF
HSF 1:4
FT
WASH
F7
F8
F14
120
86
47
34

Figure 3


M R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I -33
P A E A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I -66
N T T I A S I A A K E E G V S L E K R E A E A Y V E F T P D N N K +6
-4
-1 +1

Figure 4

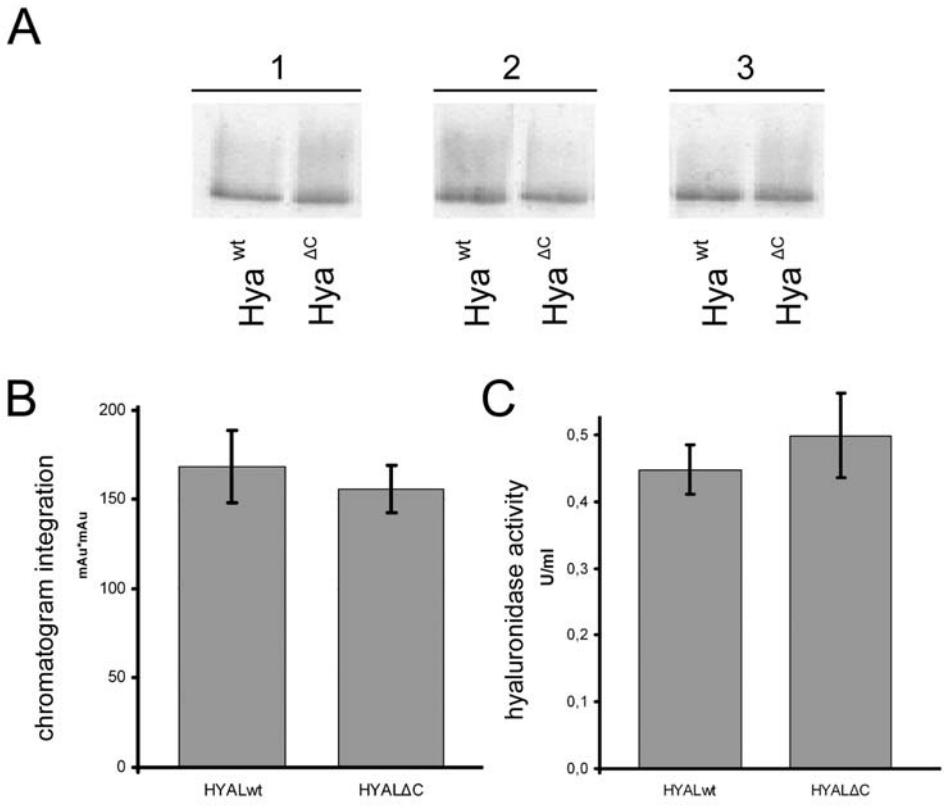
A
1
2
3
Hya wt
Hya ΔC
Hya wt
Hya ΔC
Hya wt
Hya ΔC
B
chromatogram integration
mAu*mAu
200
150
100
50
0
HYALwt
HYALΔC
C
hyaluronidase activity
U/ml
0,5
0,4
0,3
0,2
0,1
0,0
HYALwt
HYALΔC

Figure 5

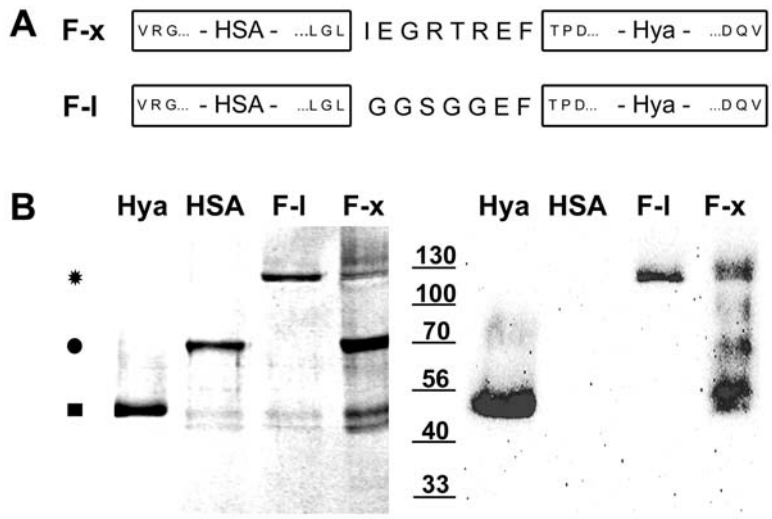
A
F-x
VRG... - HSA - ...LGL
I E G R T R E F
TPD... - Hya - ...DQV
F-l
VRG... - HSA - ...LGL
G G S G G E F
TPD... - Hya - ...DQV
B
Hya
HSA
F-l
F-x
Hya
HSA
F-l
F-x
130
100
70
56
40
33

Figure 6

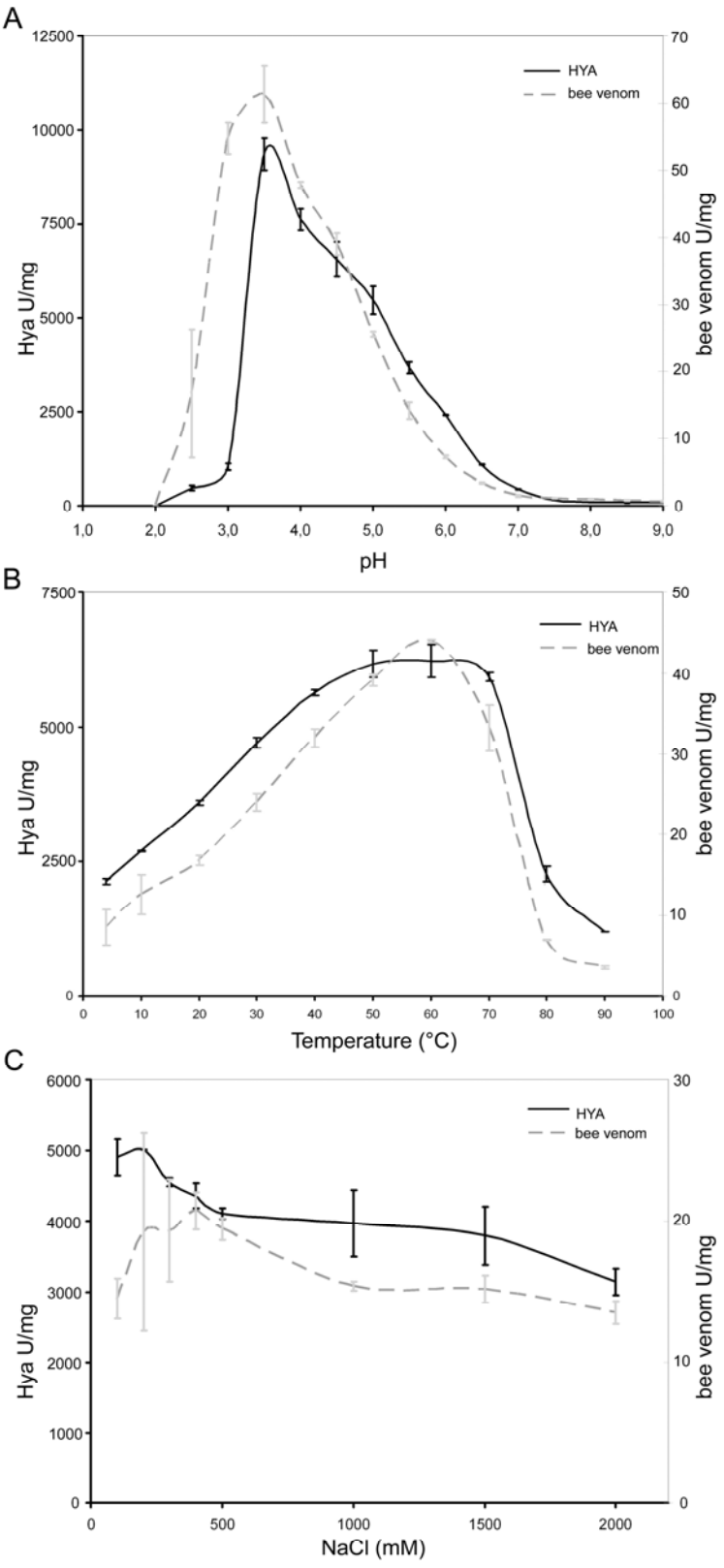
A
HYA
bee venom
Hya U/mg
bee venom U/mg
pH
B
HYA
bee venom
Hya U/mg
bee venom U/mg
Temperature (°C)
C
HYA
bee venom
Hya U/mg
bee venom U/mg
NaCl (mM)

Figure 7