TECHNISCHE UNIVERSITÄT MÜNCHEN
Fakultät für Medizin
Fachgebiet für Experimentelle Gynäkologie

Recombinant production, purification and biochemical characterization of the three human kallikrein-related peptidases KLK4, KLK8 and KLK15

Josefine Theresia Maier

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Medizin

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. E. J. Rummeny
Prüfer der Dissertation:	1. apl. Prof. Dr. V. Magdolen
	2. Univ.-Prof. Dr. M Schmitt

Die Dissertation wurde am 22.06.2012 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 30.01.2013 angenommen.

Table of contents

I Abbreviations

aa	Amino acid
AMC	7-amino-4-methylcoumarin
Amp	Ampicillin
APS	Ammonium persulfate
ATF	Aminoterminal fragment
bp	Base pair
Boc-	Di-*tert*-butyl dicarbonate
BSA	Bovine serum albumin
cDNA	Complementary deoxyribose nucleic acid
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
E. coli	Escherichia coli
e.g.	Exempli gratia (for example)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FIGO	Fédération Internationale de Gynécologie et d´Obstetrique
fl	Full length
Fl	Fluorescein
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His-tag	$Histidine_6$-tag
hK	Human kallikrein, older term to describe KLKs
HMW	High molecular weight
IPTG	Isopropyl β-D-1- thiogalactopyranoside
kDa	Kilodalton
KLK	Human kallikrein-related peptidase
LMP	Low malignant potential
LB-medium	Luria-Bertani-medium
M	Marker
min	Minute
MMP	Matrix metalloproteinase
MW	Molecular weight
Ni-NTA	Nickel-nitrilotriacetic acid
ntfl	Near to full length
ODλ	Optical density at wavelength λ
OS	Overall survival

PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pers. comm.	Personal communication
***p*NA**	*para*-Nitroanilide
PSA	Prostate-specific antigen
Q	Quencher
RKI	Robert-Koch-Institute, Germany; the institute prepares a report on cancer in Germany every two years.
RPM	Revolutions per min
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Suc-	Succinyl-
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
V	Volt

Amino acids

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	aspartic acid	P	Pro	proline
E	Glu	glutamic acid	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

II Introduction

II.1 Cancer in Germany

Cancer is a disease in which cells exhibit division beyond normal limits “uncontrolled growth”. Resulting tumors lead to intrusion and destruction of surrounding tissues (invasion) and spread to other locations (metastasis). These properties distinguish malignant cancer from benign tumors (Böcker et al., 2004). In 2006, around 440 new cancer cases per 100,000 male patients and 320 cases per 100,000 females were recorded in Germany **(fig.II.1)**. The annual number of new cancer cases is estimated at approximately 229,200 among men and 197,600 in women. Cancer can affect patients of every age, but it occurs more often in the elderly. The average age at onset is about 69 in both men and women. Malignancies develop not only from a single cause but also reflect the interplay of various risk factors. It is known that certain chemical agents, e.g. components of cigarette smoke, can cause lung cancer. Lifestyle factors such as a high-calorie diet with low-fiber nutrient content can be associated with colon cancer (Böcker et al., 2004). Other known risk factors include viral infections (HPV and cervical cancer), radiation (skin cancer), but also genetic disposition (BRCA1 and breast cancer). The most common cancer entities detected in Germany in 2006 for male patients were prostate cancer (26.2%), colon and rectum malignancies (15.8%) and lung cancer (14.2% of all cases), see **fig.II.2**. In women the most frequent malignant tumor type is breast cancer (29.3%), followed by colon and rectum malignancies (16.4%) and lung tumors (7.4% of all newly detected cases), see **fig.II.2**. Differences between sex-specific tumor entities are most likely due to diverse exposition to risk factors (Böcker et al., 2004). Mortality for some cancer types is higher than for others. For example lung cancer is the most lethal tumor in men, causing 25.7% of all cancer-related deaths **(fig. II.3)**.

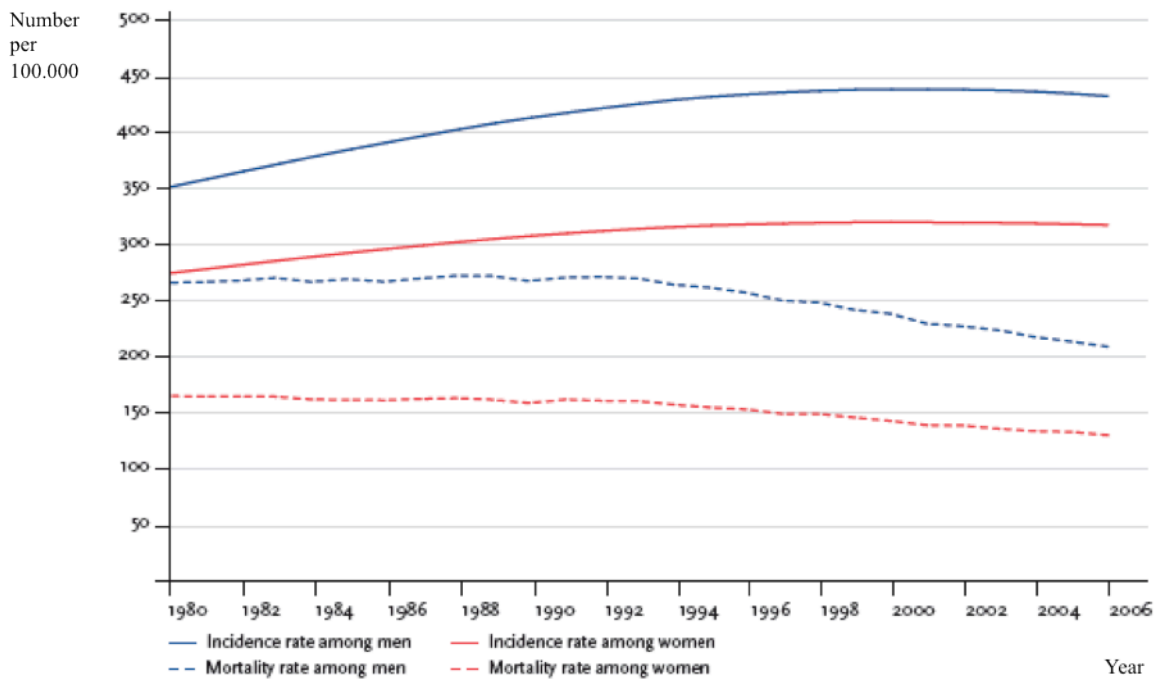


Figure II.1 Incidence and mortality rates in Germany from 1980-2006. This figure shows that incidence of malignancies has risen, whereas mortality rates have decreased over the past decades. Taken from RKI, Cancer in Germany, 2010.

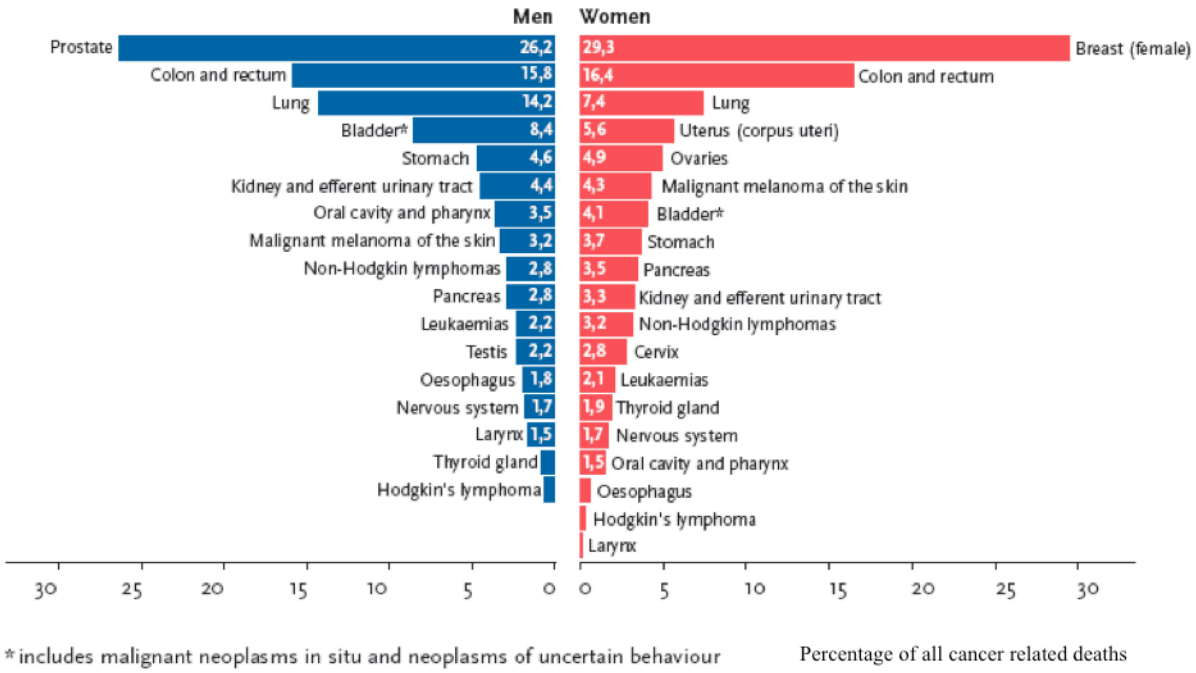


Figure II.2 Selected tumor sites as a percentage of all new cancer cases. This figure excludes non-melanoma skin cancer in Germany in 2006. Taken from RKI, Cancer in Germany, 2010.

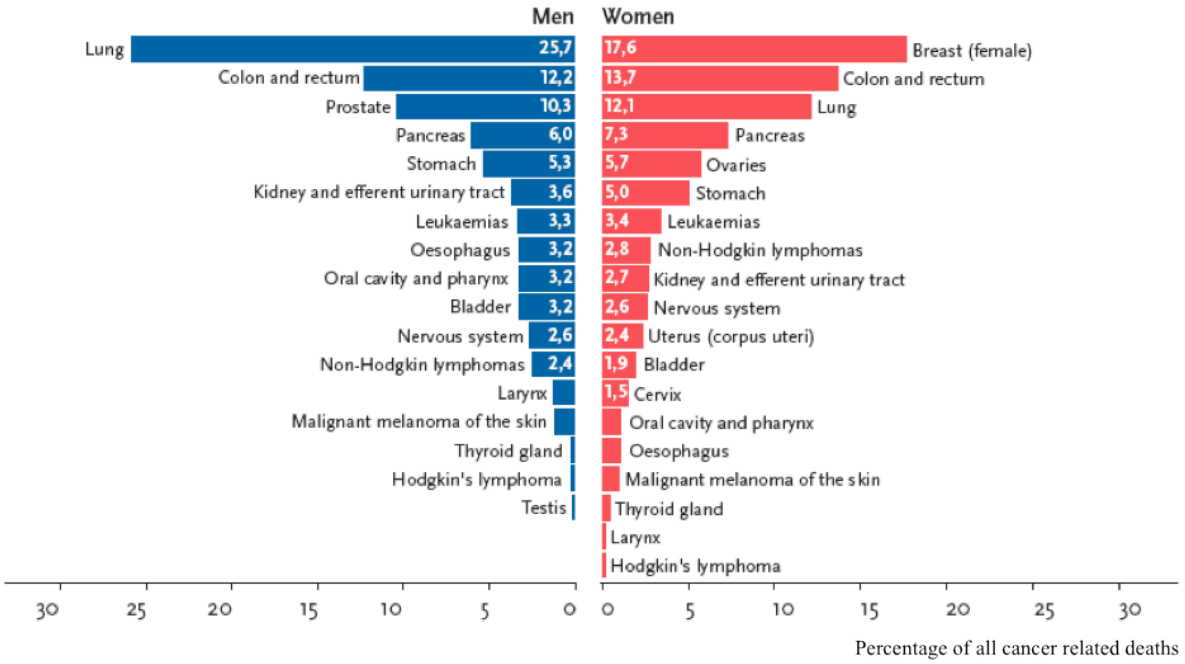


Figure II.3 Selected tumor sites as a percentage of all cancer-related deaths in Germany in 2006. Taken from the official cause-of death statistics, Federal Statistics Office, Wiesbaden (RKI, Cancer in Germany, 2010).

II.2 Gynecologic oncology

Gynecologic cancers are one of the most common tumor types in women in Germany. Gynecologic oncology is a specialized field of medicine that focuses on cancers of the female reproductive system, including breast, ovarian, cervical and endometrial malignancies. Breast cancer is the most common neoplasm in females, followed by uterine tumor at the 4th place and ovarian cancer at the 5th, see **fig.II.2** (RKI, Cancer in Germany, 2010). Cervical cancer is less common in developed countries because Papanicolaou (Pap) test screening is widely available and effective in detecting early stage cancer. This highlights the importance of research in the field of gynecology in order to develop appropriate markers for detection at early stages and treatment of tumor entities in women.

II.2.1 Ovarian cancer

Ovarian cancer is the second most common cancer of the female reproductive system in Germany; every year 8000 women are diagnosed with ovarian cancer, with a median age of disease onset of 62 years. In ovarian malignancies, epithelial cancer is the most common histologic entity, with approximately 90% of all cancers originating in the ovaries (Kiechle

et al., 2006). This malignancy has a high incidence of 14 per 100,000 women, with around 6000 patient mortalities each year from this disease in Germany **(fig.II.4)**. The exact cause of this type of tumor is not clear, but there are certain known risk factors for ovarian cancer, such as age **(fig. II.5)**, ovulatory cycles, infertility, no pregnancies, medications that induce ovulation, as well as breast cancer in the patient's history. Some protective factors are also known, including pregnancies and medications that inhibit ovulation. Ovarian cancer most frequently occurs spontaneously (90%), whereas only 5 - 10% can be attributed to genetic disposition, most commonly mutation of BRCA1, followed by BRCA2 (Kiechle et al., 2006). Ovarian carcinoma can spread by local extension, lymphatic invasion, intraperitoneal implantation, hematogenous dissemination and transdiaphragmatic passage. Intraperitoneal dissemination is the most common route and is a recognized characteristic of ovarian cancer. Malignant cells can implant anywhere in the peritoneal cavity but are most likely to implant in sites of stasis along the peritoneal fluid circulation. Early hematogenous spread is clinically unusual, whereas it is not infrequent in patients with advanced disease.

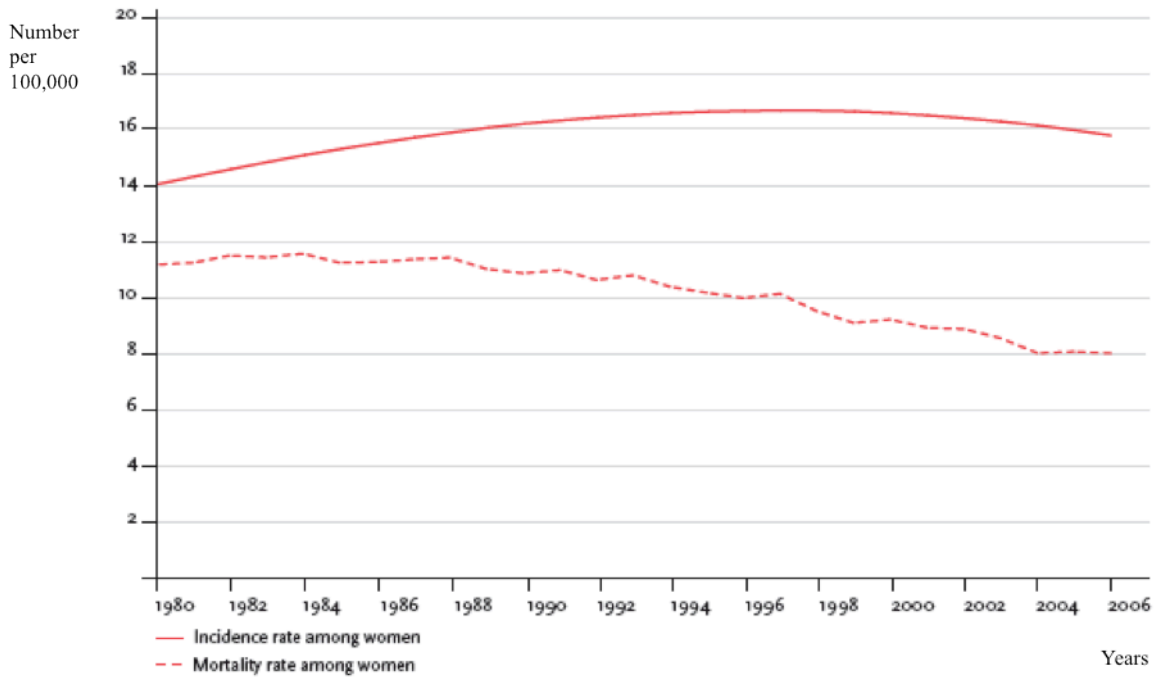


Figure II.4 Age-specific incidence and mortality rates of ovarian cancer. Incidence and mortality per 100,000 cases in Germany, 1980 – 2006. Incidence has risen, but mortality rate has fallen over the past decades. Taken from RKI, Cancer in Germany, 2010.

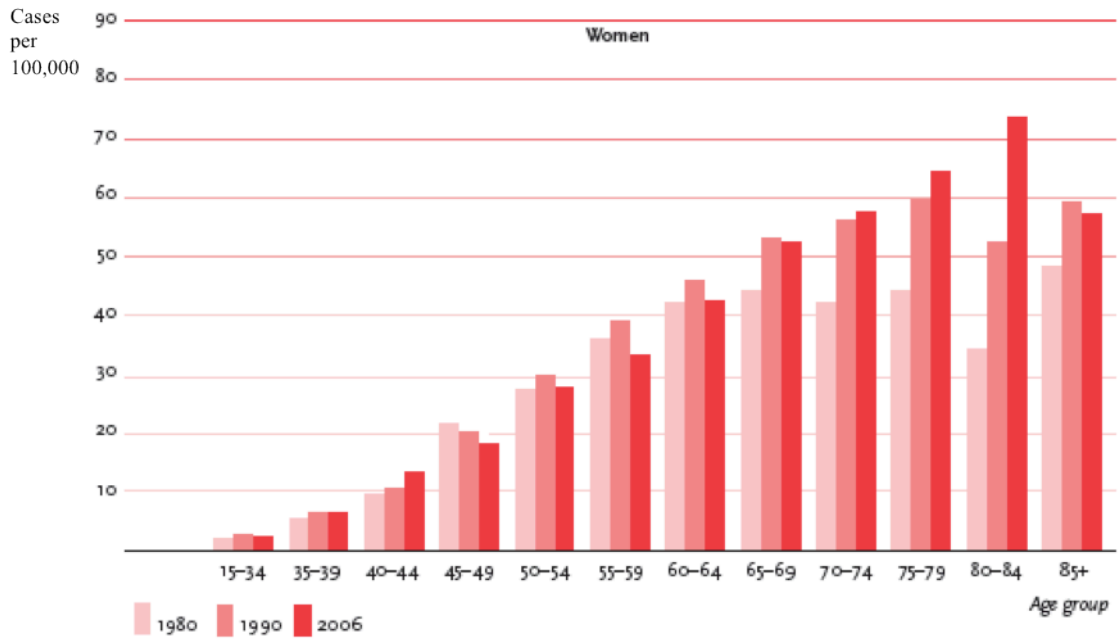


Figure II.5 Age-standardized incidence of ovarian cancer in Germany, for 1980, 1990 and 2006. Cases/deaths per 100,000. Incidence increases with age. Taken from RKI, Cancer in Germany, 2010.

Ovarian cancer is staged using the International Federation of Gynecology and Obstetrics (FIGO) staging system **(table II.1)**. It is based on information obtained after surgery, which can include a total abdominal hysterectomy, removal of (usually) both ovaries and fallopian tubes, (usually) the omentum and pelvic or peritoneal washings for cytology (Kiechle et al., 2006). Prior to surgery several examinations are performed, including abdominal ultrasound or computed tomography, chest X-ray as well as blood tests. One of the currently available biomarkers is CA-125 (cancer antigen 125), which is useful in following the response to treatment and helps to predict prognosis and possible recurrence of the tumor after therapy (Kiechle et al., 2006). However, there is still no biomarker available for specific detection of ovarian carcinoma at the early stages. There are no characteristic early symptoms specific for ovarian cancer. In 10 - 15% of all cases, the only symptom is abnormal vaginal bleeding after menopause. Later symptoms are mostly due to advanced spread of tumor cells and ascites, such as bloating, diarrhea, gas, nausea, constipation, indigestion, urinary symptoms, pelvic and abdominal pain, pain in back and legs and cachexia. As ovarian cancer does not initially present with typical symptoms, most patients are diagnosed at a stage of advanced disease. From a group of patients (n=142) who were diagnosed and treated for ovarian carcinoma between 1985 and 1999 at the Department of Obstetrics and Gynecology, Technical University of Munich, 17.6%, 7.0%, 54.9%, and 20.5% of women presented with

stage I, II, III, and IV, respectively (Dorn et al., 2007). Only 9.9% of the diagnosed tumors were well-differentiated and categorized as G1. In 60.5% of all cases, high-grade tumors (G3) were diagnosed, with poor prognosis for survival (Dorn et al., 2007). Despite this, the mortality attributable to ovarian cancer has decreased significantly in the last 20 years, even though incidence rates have remained stable in Germany, see **fig.II.4** (RKI, Cancer in Germany, 2010). The majority of patients who present with epithelial ovarian cancer respond well to the initial treatment, but will ultimately experience disease recurrence. As state-of-the-art therapy, the patient undergoes radical debulking surgery to completely remove the tumor, followed by combination chemotherapy (Dorn et al., 2007), which can palliate symptoms of disease recurrence and improve survival (Martin et al., 2009). Even so, therapeutic interventions are associated with considerable morbidity. In patients with advanced disease, radical surgery carries a 3% mortality rate. Therefore, tumor-associated biomarkers are required in order to select suitable candidates for novel primary clinical therapy approaches such as preoperative chemotherapy or a second-effort surgical approach to improve survival and reduce morbidity (Kiechle et al., 2006; Dorn et al., 2007).

In the past, various studies have revealed that protease levels are altered in cancer compared to non-cancerous disease indicating a potential role in its genesis and for metastasis. This is also true for ovarian carcinoma as shown by Clinical Research Unit (Department of Gynecology, Technical University of Munich) and others. In 1999, Graeff et al. introduced the protease uPA and its inhibitor PAI-1 as novel biomarkers that can be useful in selecting subgroups of patients with ovarian cancer in order to individualize and improve therapy and predict response (Kuhn et al., 1999). Soon it has been shown that elevated antigen levels of uPA and PAI-1 correspond with the malignancy of the tumor; levels were found to be low in benign tumors but increased significantly from LMP tumors to advanced ovarian cancer. Levels were higher in the primary tumors than in the corresponding metastases. Other proteases, such as kallikrein-related peptidases and matrix metalloproteases (MMP-2 and MMP-9) were found to be expressed aberrantly in ovarian cancer where they appear to be associated with progression from benign to advanced tumor disease (Schmalfeldt et al., 2001).

Stage I	17.6%*	Limited to one or both ovaries
	IA	Involves one ovary; capsule intact; no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
	IB	Involves both ovaries; capsule intact; no tumor on ovarian surface; negative washings
	IC	Tumor limited to ovaries with any of the following: capsule ruptured, tumor on ovarian surface, positive washings
Stage II	7.0%*	Pelvic extension or implants
	IIA	Extension or implants onto uterus or fallopian tube; negative washings
	IIB	Extension or implants onto other pelvic structures; negative washings
	IIC	Pelvic extension or implants with positive peritoneal washings
Stage III	54.9%*	Microscopic peritoneal implants outside of the pelvis; or limited to the pelvis with extension to the small bowel or omentum
	IIIA	Microscopic peritoneal metastases beyond pelvis
	IIIB	Macroscopic peritoneal metastases beyond pelvis less than 2 cm in size
	IIIC	Peritoneal metastases beyond pelvis > 2 cm or lymph node metastases
Stage IV	20.5%*	Distant metastases to the liver or outside the peritoneal cavity

Table II.1 FIGO Staging for ovarian cancer. Taken from Kiechle et al., 2006.
*Clinical characteristics of a cohort of patients (n=142) that were diagnosed and treated for ovarian carcinoma between 1985 and 1999 at the Department of Obstetrics and Gynecology, Technical University of Munich (Dorn et al., 2007). Para-aortic lymph node metastases are assessed as regional lymph nodes (Stage IIIC).

II.3 Proteases

In living systems, proteins that have served their purpose are routinely degraded to their components, so that the amino acids can be recycled for synthesis of new proteins. This protein degradation, known as proteolysis, is also essential for breaking down ingested proteins into amino acids for absorption in the gut. Furthermore, proteolytic processing regulates the activity of certain enzymes and other proteins. Proteolysis of proteins is conducted by hydrolysis of the peptide bonds that link amino acids in a polypeptide chain. Regarding their activity, proteases can be divided into two groups **(fig.II.6)**: exopeptidases, that cleave one amino acid preferentially near the C- or N-terminus of the protein and endopeptidases that hydrolyze internal peptide bonds (Light et al., 1989; Stryer et al., 2002).

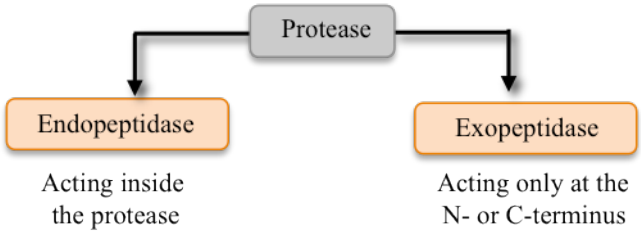


Figure II.6 Proteolytic enzymes can be divided into endopeptidases and exopeptidases. Endopeptidases act away from the termini, whereas exopeptidases act only at the N- or C-terminus. Taken and modified from Light et al., 1989; Stryer et al., 2002.

II.3.1 Serine proteases

Proteases are classified in six groups: serine, threonine, cysteine, aspartate, metallo and glutamic acid proteases by the mechanism used to cleave proteins. Serine proteases cleave peptide bonds by a nucleophilic attack of the targeted peptide bond by the hydroxyl group of the side chain of serine. The main player in this process is referred to as the catalytic triad of the active site of the protease containing serine, histidine and aspartate (Stryer et al., 2002). Serine proteases of the S1A sub-family belong to the group of endopeptidases, targeting certain sequences in the polypeptide chain, depending on the side chain of the amino acids surrounding the cleavage site (Ramsay et al., 2008). Serine proteases are known to modulate various cellular processes by selective cleavage. They are involved e.g. in blood coagulation, dietary protein digestion and wound healing. Serine proteases play an important role under various clinical conditions. For instance, mutations in the anti-coagulant protein C lead to increased activity of the enzyme and a predisposition to thrombosis. Mutations in alpha1-antitrypsin, a serum trypsin inhibitor, can predispose to severe emphysema of the lung. The active site of a protease contains the catalytic and binding site for the subsite **(fig.II.7)**. It is located in a groove on its surface and substrate specificity is determined by the interaction of binding sites on the groove of the catalytic site with amino acid side chains spanning the scissile bond that is responsible for hydrolysis. Subsites that accommodate the side-chain of single amino acid residues can be used in order to describe the specificity of a protease. They are numbered S_1, S_2 … S_n from catalytic triad to the N-terminus and S'_1, S'_2 … S'_n to the C-terminus. Respectively, accommodated amino acids are numbered P_1, P_2 … P_n and P'_1, P'_2 … P'_n (Light et al., 1989).

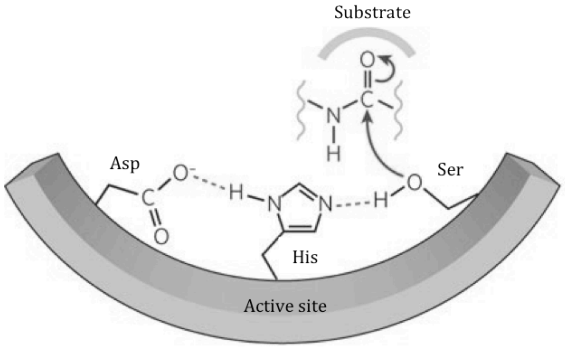


Figure II.7 Serine proteases which form a catalytic triad. The active site consists of three amino acids: histidine, serine and aspartic acid, the catalytic triad. Serine proteases modify peptide bonds by a nucleophilic attack of the targeted peptide bond by the hydroxyl group of the side chain of serine. By this, the substrate is bound to the active site. Taken and modified from Erez et al., 2009.

II.3.1.1 Trypsin-like serine proteases and zymogens

The general cleavage site specificity of serine proteases is determined by the residue that occupies the S_1 site six amino acids before the serine of the catalytic triad, dividing them into chymotrypsin- and trypsin-like serine proteases **(table II.2)**. Trypsin-like serine proteases generally contain the negatively charged aspartate in this position, allowing them to cleave specifically after positively-charged arginine or lysine residues. Chymotrypsin-like proteases, with a hydrophobic amino acid residue in this subsite, hydrolyze peptide bonds C-terminal to large hydrophobic amino acids like tryptophan. A large, non-polar residue specifies elastase to cleave after small hydrophobic amino acids like alanine. Most trypsin-like serine proteases are secreted as inactive enzyme precursors in order to prevent auto-digestion of cells and tissue. Their inactive form is activated by proteolytic cleavage of one or a few peptide bonds (Stryer et al., 2002). Proteolytic cleavage leads to rearrangement of the region encompassing the active site and, as a consequence, to the formation of the catalytic triad. This can be observed in diverse processes as a way to control the activity of proteases **(fig.II.8)**. Trypsinogen is activated by enterokinase or trypsin. In turn, trypsin then activates a host of other zymogens, leading to the digestion of proteins. Hydrolysis of a single peptide bond by trypsin converts the zymogen chymotrypsinogen into active chymotrypsin. In the blood clotting cascade, zymogens are cleaved by activated enzymes, leading to the cleavage of the next precursor. At the bottom of the cascade, fibrinogen is converted to fibrin by thrombin, resulting in the insoluble fibrin fibers that form the clot (Stryer et al., 2002). Other protease-substrate binding regions outside of the active site, such as specificity pockets P1-P1' or other exosites, can influence the specificity of serine proteases. Trypsin can be considered

a non-specific protease as it cleaves after almost any Lys or Arg residue, whereas thrombin has much higher specificity as it cleaves only fibrin and a few other selected substrates.

	Trypsin	**Chymotrypsin**
Residue S1 pocket	Aspartate	Serine
Substrate specificity	Arginine or lysine	Large hydrophobic amino acids (phenylalanine, tryptophan, tyrosine)
Activity	Trypsin-like	Chymotrypsin-like
Zymogen	Trypsinogen	Chymotrypsinogen
Activation	Secreted from pancreas, enters small intestine; activated by enterokinase and auto-activation; subsequently activates other zymogens	Activated through cleavage by trypsin

Table II.2 Serine proteases trypsin and chymotrypsin. This table shows their cleavage site specificity and physiological role, from Stryer et al., 2002.

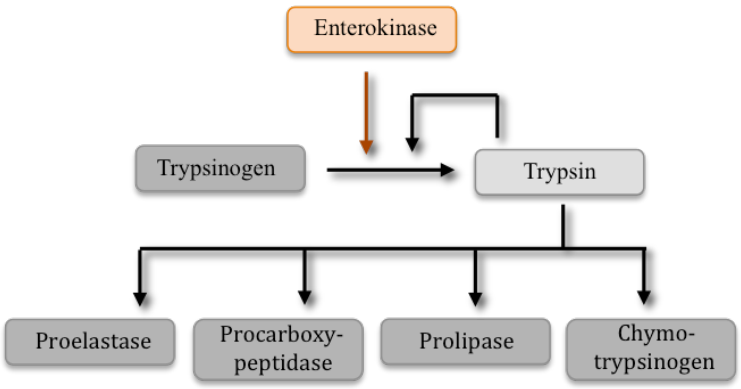


Figure II.8 The interplay of enterokinase, trypsinogen and trypsin. Trypsinogen, secreted by the pancreas into the duodenum, is activated by enterokinase and is subsequently able to perform auto-activation. Hereafter, trypsin cleaves further zymogens involved in digestion, such as proelastase, chymotrypsinogen, prolipase and procarboxypeptidase converting them to active enzymes. Taken and modified from Stryer et al., 2002.

II.3.2 Kallikrein-related peptidases (KLKs)

Human tissue kallikreins, also known as KLKs or kallikrein-related peptidases, are a subgroup of extracellular serine proteases and belong to the S1 family of the clan SA (Emami et al., 2007). They are expressed in various tissues and cell types and implicated in a wide range of normal physiological functions. By selective cleavage of specific substrates,

KLKs are involved in processes such as semen liquefaction, skin desquamation and neuronal plasticity. There is also evidence that KLKs interact with each other in terms of activation and form a network (Paliouras et al., 2003; Schmalfeldt et al., 2001; Emami et al., 2007). Interestingly, KLKs, for which their substrates and functions are still poorly characterized, display aberrant expression patterns in a number of cancer types. For several kallikreins a clear correlation between expression and certain types of cancer have been verified. Further study could help to establish therapies that target KLKs in cancer treatment (Yousef et al., 2003).

II.3.2.1 The beginning of the kallikrein era

In 1930, the term "kallikrein" was introduced by Heinrich Kraut. Five years earlier the surgeon Emil-Karl Frey had discovered that the injection of human urine into dogs led to the reduction of arterial blood pressure. He assumed that a substance in the urine had a cardioactive and vasoactive effect. This substance was later called "kallikrein" (in Greek the "kallikreas" is the pancreas), as it was thought to originate from the pancreas where it was found at abundant levels. In 1937, Eugen Werle characterized the function of kallikrein as a proteolytic enzyme that cleaves kininogen. This results in kallidin, a hormone that acts locally to induce vasodilation and contraction of smooth muscle.

II.3.2.2 Progress in the KLK research since the 1980s

Today, kallikreins are divided into two major categories: plasma kallikrein and tissue kallikreins, differing significantly in molecular weight, substrate specificity, immunological characteristics and gene structure. Until the end of the 1980s, the KLK family was assumed to consist of only three members, namely KLK1, the pancreatic/renal kallikrein, KLK2 or human glandular kallikrein 2, and KLK3, also known as prostate-specific antigen (PSA) (Yousef et al., 2001; Clements et al., 2008). Since 1994, other proteases with similar activity to the known KLKs have been found and further characterized (Borgoño et al., 2004). In all, 15 human kallikrein-related peptidases have been identified. They are encoded by the human tissue kallikrein gene family, located on chromosome 19q13.4 (Shaw et al., 2004), representing the largest contiguous family of proteases in the human genome.

a **b**

Figure II.9 Logo of the E. K. Frey – E. Werle Foundation of the Henning L. Voigt Family (fig.a) and Emil-Karl Frey, Eugen Werle and Heinrich Kraut (from left to right, fig.b). The surgeon Emil-Karl Frey discovered KLK1 first as being cardioactive and vasoactive. The term "kallikrein" was introduced by Heinrich Kraut some years later. In the 1940s, Eugen Werle KLK1 as proteolytic enzyme cleaving kininogen. Taken from the E. K. Frey – E. Werle Foundation.

II.3.2.3 Genomic organization of KLKs

The human kallikrein family is known to consist of 15 genes, located on chromosome 19q13.4 as a cluster encompassing approximately 300 kbp **(fig.II.10)**. KLK genes share common features, they range between 4.4 and 10.5 kbp in size and are composed of five coding exons and one untranslated exon (Shaw et al., 2006). Phylogenetic analysis confirms that mammalian KLK genes are highly homologous, suggesting a conserved function of the encoded KLKs. KLK1 to 3 form a subgroup of closely-related proteases. Based on phylogenetic analysis, the KLK proteases can be sub-divided into groups of KLK4/5/7, KLK6/13/14, KLK8/ 10/12, and KLK9/11/15 (Debela et al., 2006). Many alternative splice variants of KLKs have been identified. KLK proteases are expressed as single-chain pre-pro-serine proteases. During secretion the pre-domain (signal sequence, 16 to 20 amino acids) is cleaved off from the N-terminus of the protein. Pro-domains, containing 4 to 9 amino acids (except proKLK5 with 37 amino acids), are cleaved off at trypsin-like cleavage sites, after arginine or lysine residues leading to the activation of the proteases (Clemets et al., 2008; Emami et al., 2007). ProKLK4 and proKLK13 are exceptions of this pattern, they are activated by still unknown proteases by cleavage of the pro-peptide after glutamine and phenylalanine, respectively (Goettig et al., 2010). The mature KLK contains a conserved catalytic triad of histidine (His 57), aspartic acid (Asp 102) and serine (Ser 195). Protein folding is stabilized through 5 to 6 intermolecular disulfide bonds.

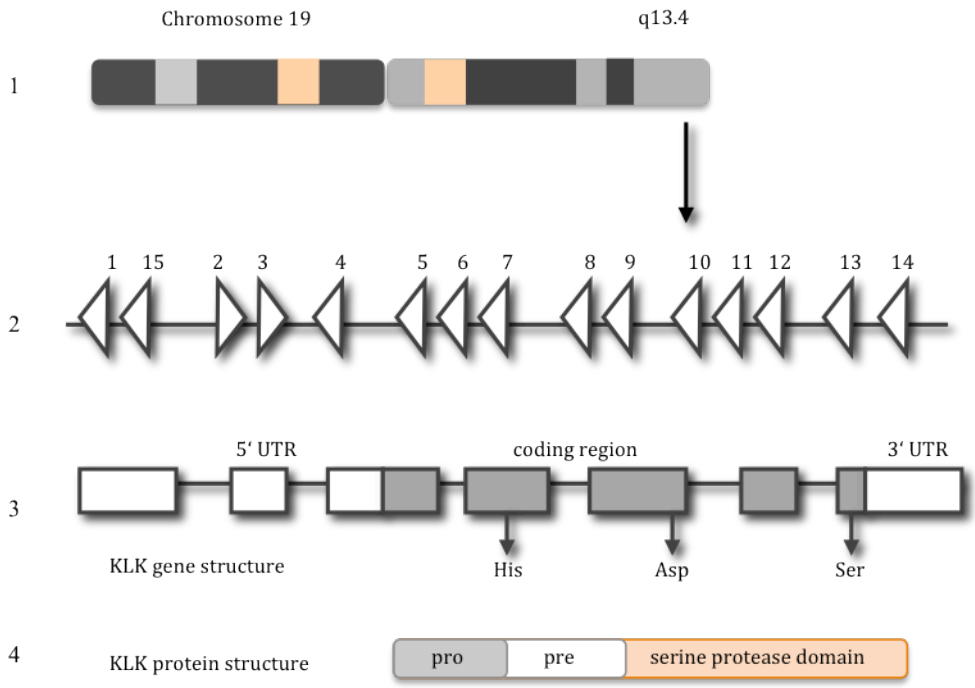


Figure II.10 The human tissue kallikrein gene cluster. The genes encoding tissue KLKs are localized in tandem on human chromosome 19q13.4 and constitute a serine protease subfamily of 15 members (picture 1). Each arrowhead on picture 2 indicates the direction of transcription of the corresponding gene. Most KLK genes contain five coding exons and one or two untranslated 5′ exons (shown in white boxes in picture 3). His (histidine), Asp (aspartic acid) and Ser (serine) represent the catalytic residues located in the active site. KLKs are synthesized as inactive pre-proKLKs. Upon proteolytic removal of the pre-domain, activation of the pro-peptide or zymogen is required for conversion to an active (mature) enzyme. In this schematic representation of KLK gene structure, boxes indicate exons and lines indicate intervening introns. Modified from Pampalakis et al., 2001.

II.3.2.4 Expression and regulatory mechanisms of KLKs

KLKs were found to be expressed both at mRNA and protein levels by different cell types in various tissues, implicating a wide range of functions of these proteases (Emami et al., 2007). Expression is believed to be regulated by both hormones and epigenetic factors, such as DNA methylation and histone modification. Some KLK genes contain hormonal response elements (HRE), suggesting a regulation by steroid hormones. Patterns of up- and downregulation of KLK expression occurs in a number of diseases including some hormone-dependent carcinomas. For example, it has recently been shown that in breast cancer the expression of KLK10, 11, 13 and 14 is dependent on dihydro-testosterone (DHT) and norgestrel (Emami et al., 2007).

II.3.2.5 Molecular features of KLKs

As serine proteases, kallikrein-related peptidases hydrolyse substrates through a nucleophilic attack directed by a serine residue located in the active site (Murray et al., 2006). Most kallikrein-related peptidases contain the negatively-charged aspartate (and in case of KLK15 glutamate) at their S_1-position, indicating a trypsin-like activity (KLK1, 2, 4, 5, 6, 8, 10, 11, 12, 13, 14 and 15). KLK3, 7 and 9 have serine, asparagine and glycine, respectively, in S_1 and present chymotrypsin-like substrate specificity (Pampalakis et al., 2001; Debela et al., 2006). Activity of kallikrein-related peptidases is regulated by inactivation mechanisms through internal cleavage leading to degradation, mediated by either the KLK itself or other proteases. Numerous inhibitors for KLKs have been described, such as endogenous serpins and zinc ions that control their activity (Emami et al., 2007). KLK1, the "true tissue kallikrein", is involved in various processes mediated through kinin, such as blood pressure regulation, smooth muscle contraction, neutrophil chemotaxis and pain induction. Furthermore, there is evidence that KLK1 might act independently of the kinin pathway, through growth factors and other substrates. KLK2, 3, 5 and 11 are known to function in a proteolytic network in seminal plasma. KLK4 plays a role in enamelogenesis by cleavage of enamelin (Emami et al., 2007). KLK6, 10 and 13 were found to activate some prohormones in the islets of Langerhans in the pancreas (Emami et al., 2007). KLK6 and 8 may be involved in myelination and synaptogenesis in the central nervous system (Emami et al., 2007).

II.3.2.6 KLKs form networks: Semen liquefaction

To initiate a proteolytic cascade, an activated protease cleaves other protease zymogens, activating them for downstream functions, and leading to the rapid amplification of the original signal. Serine proteases constitute a subgroup of proteases known to participate in proteolytic cascades (Pampalakis et al., 2001). A well-studied process is semen liquefaction. Here KLK3 or PSA is known to play a key role **(fig.II.11)**. This particular protease network is, in fact, an example for a well-analyzed physiological cascade with KLKs involved. It is a highly-regulated proteolytic pathway, initiated by ejaculation, which activates the zymogen proKLK3. At the time of ejaculation, the epididymal fluid containing sperm is mixed with prostatic fluid. The latter is composed of various proteases such as KLK2, 3, 4, 5, 8, 11, 12, 14, 15 (Yousef et al., 2001). An important feature of the prostatic fluid is its high concentration of zinc-ions (Zn^{2+}), which inactivate KLKs by reversible allosteric binding.

After ejaculation, KLKs release Zn^{2+} and are reactivated. It has been demonstrated that certain KLKs are able to activate proKLK3, such as KLK2, 4, 5 and 15. Others, like KLK5, are responsible for internal cleavage and degradation of KLK3 and therefore act as a regulator of semen liquefaction. Additionally, it has been shown that the KLK network crosstalks with plasmin, TGF beta-1 and protein C-inhibitor. After ejaculation, semen proteins such as semogelin I and semogelin II aggregate to form a gelatinous mass containing trapped spermatozoa. In 5 to 20 min, the ejaculate is liquefied and the spermatozoa released due to the activation of KLK3 and other proteolytic enzymes (Pampalakis et al., 2007).

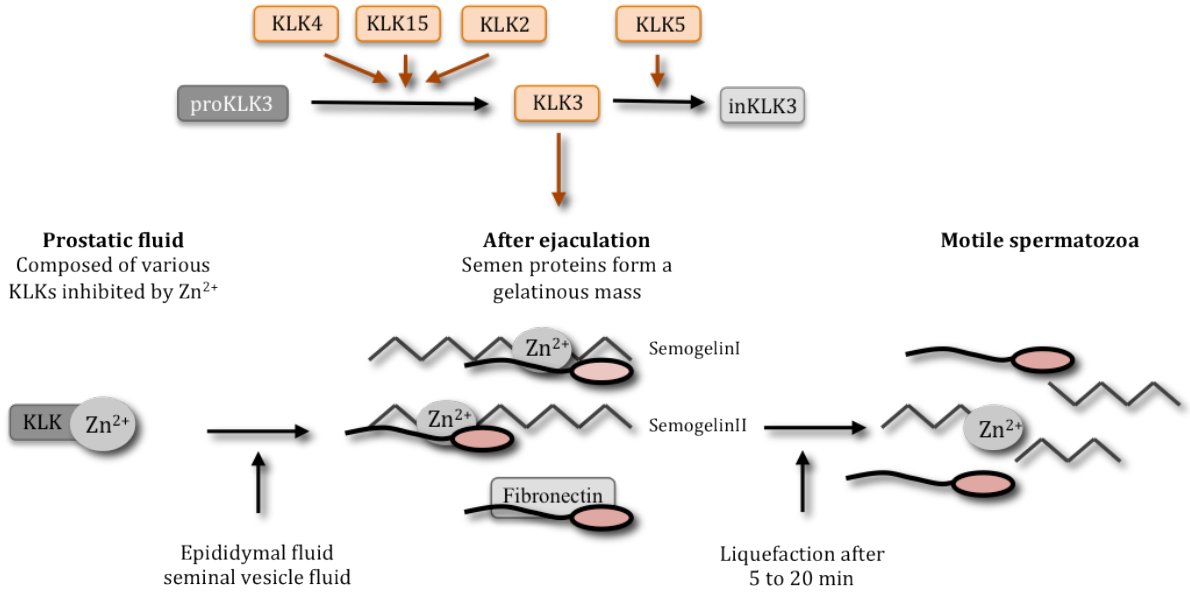


Figure II.11 Semen liquefaction cascade. The cascade involves several KLKs, that are present in prostatic fluid, where they are inhibited by Zn^{2+}. At the time of ejaculation, mixing of static fluid with epididymal and seminal vesicle fluids, results in Zn2+ redistribution and dilution and consequently in activation of KLKs. As a result the semen forms a gelatinous mass. Upon the activation of KLKs, especially KLK3, the aggregated semen proteins such as Semogelin are cleaved and the semen becomes more liquid leaving the spermatozoa motile. InKLK indicates inactive KLK. Modified from Pampalakis et al., 2001.

II.3.2.7 Pathological functions of KLKs

Overexpression and overactivation of kallikrein-related peptidases lead to excessive proteolysis, found in various pathological conditions such as cancer and non-cancerous diseases. The kallikrein-kinin system is involved in many disease processes including hypertension, inflammation, pancreatitis, renal disease, as well as malignant tumors (Yousef et al., 2001). Carcinogenesis is a complex process that includes alterations in gene structure

and expression, it results from alterations in the DNA, mRNA and protein level. Certain KLKs have been found to be overexpressed in different cancer types and are thus suspected to play a role in the development of malignancies (Debela et al., 2006). *In vitro* studies revealed that KLKs are able to process growth factor binding proteins and cleave components of the extracellular matrix (ECM), indicating that they might play significant roles in tumor metastasis and invasion, and may promote tumor angiogenesis (Pamplakis et al., 2007; Emami et al., 2007). There is evidence that KLKs, by affecting insulin-like growth factor (IGF), IGF binding proteins (IGFBPs), the plasminogen-activation system and proteinase-activated receptors (PARs), may lead to tumor growth and disease progression (Emami et al., 2007). In addition, KLKs have been found to be involved with matrix metalloproteases (MMPs), cAMP, Akt/PKB and vascular endothelial growth factor (VEGF) in inducing angiogenesis (Emami et al., 2007). There is growing evidence indicating that certain KLKs are part of an enzymatic cascade pathway that is activated in tumors such as ovarian, prostatic and pancreatic cancer (Paliouras et al., 2007). Interestingly, some KLKs appear to indicate poor clinical prognosis, while others seem to be an indication of either favorable or unfavorable prognosis depending on the cancer type (Mavridis et al., 2010). Therefore, many KLKs have been identified as promising diagnostic/prognostic biomarkers for several cancer types (Borgoño et al., 2004). Furthermore, KLKs might be useful as therapeutic targets with specific synthetic inhibitors, as drugs for tumor targeting or in gene therapy.

KLK4, 5, 6 and 7 were found to be overexpressed in ovarian carcinoma at the mRNA and/or protein level, whereas in normal ovarian tissue they are only moderately expressed (Debela et al., 2008). An *in vivo* tumor model showed that overexpression of KLK4, 5, 6 and 7 leads to an increased malignant phenotype of ovarian cancer cells, suggesting these proteases may be involved in ovarian tumor establishment, growth and/or spread. Elevated levels of KLK4 and 5 mRNA are associated with poorer disease prognosis. Additionally, increased KLK7 mRNA seems to be related to poorer prognosis, especially in patients with lower grade disease and those who were treated optimally. Tumor tissues that express high levels of KLK6 were found in patients with shorter disease-free and overall survival. These findings indicate that KLK4, 5, 6 and 7 play an important role in ovarian cancer development and may be useful as clinical biomarkers (Paliouras et al., 2007). Interestingly, overexpressed KLK genes are often found to be downregulated in breast, prostate and testicular tumors. Elevated levels of KLK7 mRNA are related to poorer prognosis in ovarian cancer but with good prognosis in breast cancer, suggesting that KLK7 plays different roles depending on cancer types (Paliouras et al., 2007).

II.3.2.8 KLK4

Kallikrein-related peptidase 4 **(fig.II.12)** is also known as prostase or enamel matrix serine protease and S01.251 from clan PA(S) according to the MEROPS database. The protease has trypsin-like substrate specificity, preferring arginine or lysine at P_1. It is predominantly expressed in the prostate, but is also detectable in other tissues such as breast, endometrium, skin, thyroid and the salivary gland.

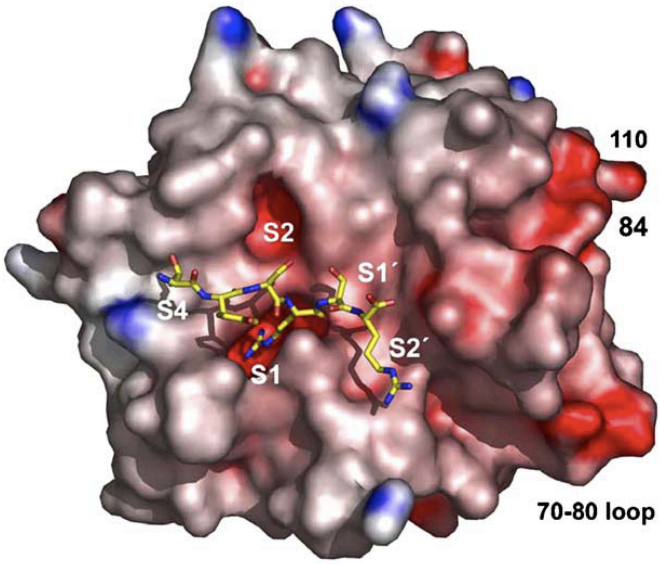


Figure II.12 KLK4 molecular surface with the modeled hexapeptide Thr-Tyr-Ser-Arg-↓-Ser-Arg. The surface of KLK4 is colored according to the electrostatic surface potential, demonstrating the acidic character (indicated in red) of the S_1 pocket and the S_2 groove, which is unique among the tissue kallikreins. Interestingly, there is a strong negative potential around the metal binding site in the 70–80 loop, around Glu84, and around the short loop at Glu110. These regions may contribute to a novel substrate binding exosite (Beaufort et al., 2006). The hexapeptide Thr-Tyr-Ser-Arg-↓-Ser-Arg is derived from the natural KLK4 substrate uPAR-D1, occupying the subsites S_4 to S_2' (Beaufort et al., 2006).

It has been shown to be significantly overexpressed in both prostate and ovarian cancers (Beaufort et al., 2006). KLK4 is also detectable in ovarian tissue under normal conditions. The protease is mainly a secreted protein. Splice variants lacking an intact or complete catalytic triad have been described (Debela et al., 2006). It is worth noting that high KLK4 mRNA levels were shown to be an independent indicator of unfavorable prognostic outcome for patients with well- and moderately-differentiated ovarian cancers (Mavridis et al., 2010), suggesting this protease might be a novel biomarker. The crystal structure of KLK4 has been resolved, facilitating further research on the substrate specificity and function of the protease (Debela et al., 2008). Recently, it was shown by our group, using immunohistochemical

analysis, that KLK4 is highly expressed in human colon adenocarcinomas in comparison to normal epithelia, which displayed low level expression of the protease. Using a colon cancer cell model, it was found that KLK4 affects proteinase-activated receptors (PARs), which are involved in human colon cancer cell proliferation (Gratio et al., 2009).

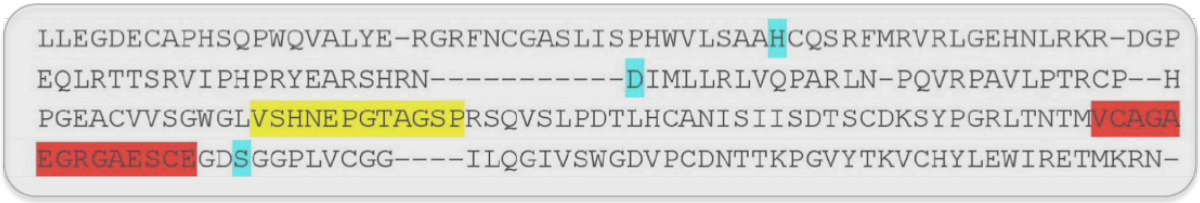
II.3.2.9 KLK8

Kallikrein-related peptidase 8, also known as neuropsin, was found to be involved in cancer and degenerative disease. KLK8 is listed as S01.244 from clan PA(S) according to the MEROPS database. The protease displays a trypsin-like substrate specificity. In 1995, the KLK8 gene was first cloned from mouse hippocampus cDNA and found to be involved in brain function. The human ortholog encodes for a 250 amino acid pre-pro-enzyme containing the catalytic triad of histidine, aspartate and serine (Kishi et al., 2006). KLK8 seems to be involved in various diseases and cancer types: it was found to be overexpressed in colon, cervical and ovarian cancer. Interestingly, it has been shown that KLK8 expression is downregulated in breast cancer, indicating that the protease performs diverse tasks (Paliouras et al., 2003). It is worth noting that increased KLK8 concentrations were found in the serum from 60% of ovarian cancer patients compared to controls, suggesting that the protease may be a good candidate for a serum biomarker. Additionally, there is evidence that KLK8 is associated with development and metastasis of ovarian carcinoma, as reported for other proteases. It was also shown that the KLK8 gene is overexpressed in cervical cancer cell lines but not in normal epithelial cells. Furthermore, it has been demonstrated that cervical tumors express KLK8, suggesting it as a potential biomarker for monitoring response to therapy as well as detection of early recurrence after treatment. KLK8 may potentially serve as a novel target antigen for cervical tumor disease (Cane et al., 2004).

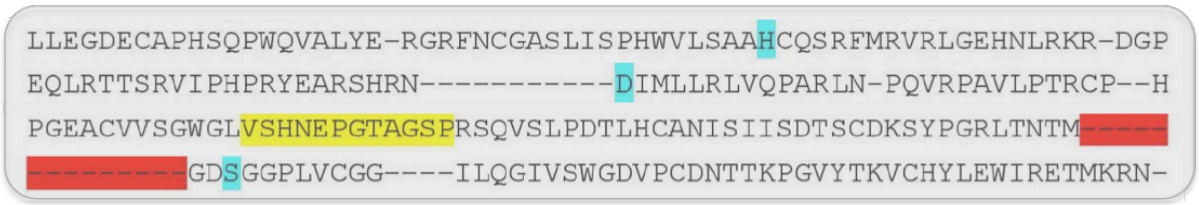
II.3.2.10 KLK15

Like most KLK genes, the KLK15 gene is formed by five coding exons and encodes for a serine protease, having a molecular weight of approximately 28 kDa. The KLK15 gene shares a high degree of structural similarity with KLK3. It is regulated by steroid hormones, possibly through the androgen receptor (AR), located between the KLK1 and KLK3 genes. Kallikrein-related peptidase 15, also known as prostinogen (Takayama et al., 2001), is

primarily expressed in the thyroid gland and has been found to a lower extent in the prostate, salivary, and adrenal glands, as well as in the colon, testis and kidney, suggesting a variety of physiological functions of the protease (Yousef et al., 2001). Compared to other serine proteases and members of the KLK-family, KLK15 has a unique insertion of 10 amino acids **(fig.II.13)**. Studies showed that this insertion might be located at the surface of the protease, forming a flexible loop **(fig.II.14)** that might interact with substrates of KLK15. Furthermore, a glutamate-residue (Glu 189) at the active site, might affect the P_1-residue, leading to a possibly smaller substrate pocket and limited substrate specificity for KLK15. Alternatively spliced forms of KLK15 are also known. The laboratory of the Clinical Research Unit (Department of Gynecology, Technical University of Munich) detected and cloned a novel splice variant of KLK15 that was found to be expressed in samples of ovarian carcinoma tissue. This novel variant, lacking 14 amino acids compared to full length KLK15 (KLK15 fl), is called KLK15 near to full length (KLK15 ntfl). The deletion by splicing at the mRNA level leads to an in-frame truncation including the glutamate 189 near the serine residue of the active site (Ser 195) **(fig.II.13 and II.14)**. KLK15 has previously been shown to hydrolyse proKLK8 and 14. Interestingly, KLK15 has the highest efficiency to hydrolyse against its own pro-peptide sequence, indicating its high autocatalytic activity (Yoon et al., 2009). Studies indicate that patients with KLK15-positive ovarian cancer have reduced progression-free survival (PFS) and overall survival (OS) compared to KLK15-negative patients **(fig.II.15)**, suggesting that KLK15 may be involved with more aggressively growing ovarian tumors (Yousef et al., 2003). There is evidence that KLK15 might play a role in ovarian cancer, as KLK15 expression levels were significantly higher in cancerous compared with benign tissues. Patients with higher mRNA expression of KLK15 showed both reduced progression-free and overall survival **(fig.II.15)**, suggesting KLK15 as an independent marker of unfavorable prognosis for ovarian cancer (Yousef et al., 2003). Studies have shown that KLK15 is involved in prostate cancer, indicating its potential as a diagnostic and/or prognostic marker for prostate cancer. The expression of KLK15 was shown to be up-regulated by steroid hormones in a prostate cancer cell line. In addition, at the mRNA level, KLK15 is also up-regulated in prostate cancer in comparison to normal prostatic tissue. Furthermore, KLK15 was found to be up-regulated by androgens and progestins in breast cancer cell lines, indicating a possible connection with this malignancy as well (Yousef et al., 2001).



a



b

Figure II.13 Amino acid sequence of KLK15 fl and its splice variant KLK15 ntfl. Both proteases have a unique insertion (yellow box) compared to other KLKs. The amino acid sequence lacking in KLK15 ntfl is marked with a red box. Blue boxes indicate the catalytic triad. Amino acid sequences are given in single letters. Fig. a depicts KLK15 fl, fig. b KHK15 ntfl.

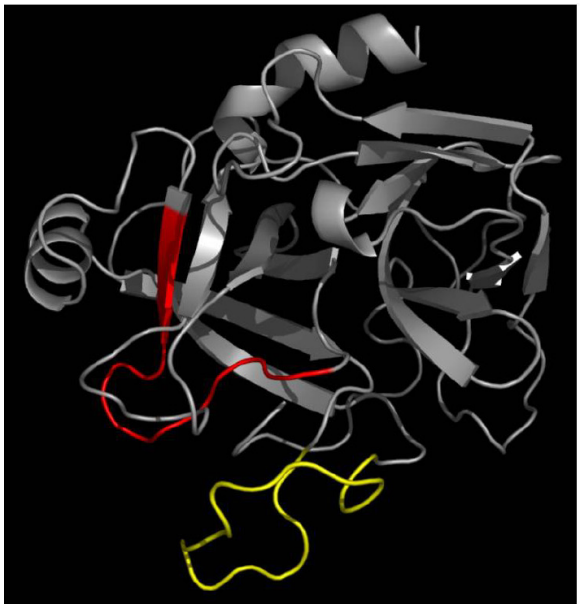


Figure II.14 Structural model of KLK15. The yellow section indicates the unique insertion of KLK15 fl and ntfl. The amino acid sequence lacking in KLK15 ntfl is indicated in red. Kindly provided by S. Sperl.

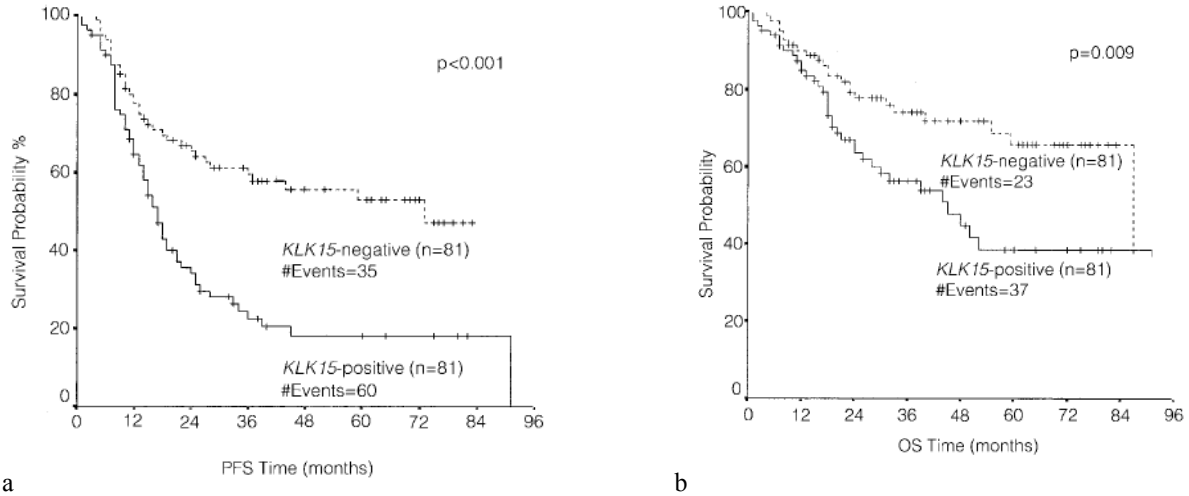


Figure II.15 Kaplan-Meier survival curves of patients with KLK15-positive and KLK15-negative ovarian tumors. KLK15 expression was measured at mRNA level. Fig. a indicates that KLK15-positive patients have a significantly reduced progression-free survival (PFS) compared to KLK15-negative patients (p value <0.001). Fig. b shows that overall survival (OS) in patients with KLK15-negative ovarian tumors is higher than in patients with KLK15-positive ovarian cancer with a probability of p=0.009. These findings indicate that KLK15 may be involved with more aggressively growing ovarian tumors. Taken from Yousef et al., 2003.

III Aims of this project

The primary aim of this project was to recombinantly produce and purify synthetic pro-enzyme forms of three human proteases: KLK4, KLK8 and KLK15. The pro-peptide harbors a histidine$_6$-tag, which can be used for purification followed by an enterokinase cleavage site. After activation of the purified and refolded proteins by enterokinase, the resulting mature enzymes should be biochemically analyzed.

In the case of KLK15, a splice variant (KLK15 ntfl) exsists, which is expressed in ovarian cancer. This variant was to be produced and purified and investigated for its potential enzymatic activity.

Finally, it was planned to expand the existing knowledge concerning the (patho-) physiological role of KLK4, KLK8, and KLK15, especially with regard to their interaction with potential cancer-related targets, such as the urokinase-type plasminogen activator (uPA) and proteinase-activated receptors (PARs).

IV Material and methods

IV.1 Materials and equipment

IV.1.1 Buffers and solutions

	Composition
1% agarose gel	0.4 g agarose added to 40 mL 1xTBE-Puffer, heated until the granules are dissolved, after cooling down to less than 50°C, evaporated amount of water is added, solution poured into mold, 20 µL ethidium bromide added
2xTY medium	1 l contains Bacto-Tryptone 16 g, Bacto-Yeast Extract 10 g, NaCl 5 g; dissolved in deionized water, pH adjusted to 7.0 with 5 N NaOH, autoclaved (Bacto-Tryptone and Bacto-Yeast Extract by BD, USA)
Amicon storage buffer	25% ethanol, 100 mM NaOH
Ampicillin	270 mM stock solution containing 100 mg/mL; MW 371.4 g/mol, storage at -20°C
Benzamidine Sepharose™ 6B	Benzamidine covalently bound to sepharose 6B, in a suspension of 6% agarose in 20% ethanol; p-aminobenzamidine (PABA) is a synthetic inhibitor of trypsin (GE Health Care, Uppsala, Sweden)
Coomassie 0.1%	Coomassie brilliant blue R-250 (Serva, Germany), 0,1% dissolved in 10% acetic acid
Cracking buffer	1% 2-mercaptoethanol, 1% sodium dodecyl sulfate, urea buffer 8 M
Developing solution for Silver-staining	25 mg/mL Na_2CO_3, add 0.1% formaldehyde solution 37% shortly before usage
DNA marker	DNA 1 kb DNA ladder 25_2030 (peqlab Biotechnology GmbH, Germany)
dNTPs	2.5 mM solution, containing 10 µL dATP, 10 µL dTTP, 10 µL dGTP, 10 µL dCTP, 360 µL H_2O
Dpn I	Restriction endonuclease (New England BioLab, USA)
Dpn I endonuclease	Target sequence: 5´-Gm6ATC-3´, a specific endonuclease for methylated and hemimethylated DNA, it is used to digest the parental DNA template; taken from the QuikChange® II Site-Directed mutagenesis Kit, Catalog #200523 and #200524, storage at -20°C (Stratagene, LaJolla, USA)
EcoR I	Restriction endonuclease, cuts nucleic acid sequence 5' G↓AATTC 3', complementary sequence 3' CTTAA↓G 5', 20 U/µL, stored at -20°C (New England BioLab, USA)
Elution buffer for benzamidine columns	100 mM glycine, 500 mM NaCl, pH 3.5 and Tween-20 0.005%, autoclaved, storage at room temperature
Enterokinase	Light Chain, porcine Cat. No. : Z01003, Size : 100 IU, 2 U/µL, theoretical MW:

	21,880 Da, actual MW on SDS-PAGE circa 40,000 Da, storage at -20°C (GenScript, USA)
Enterokinase removal kit	Consists of an antibody directed against enterokinase coupled to agarose, provided in 50% (v/v) suspension containing 0.01 M phosphate buffered saline and 15 mM sodium azide; to capture 1 U of porcine enterokinase from the solution 50 µL of 50% suspension was utilized, storage at 4°C (Sigma, USA)
Fixation solution for SDS-PAGE stains	45% ethanol, 10% acetic acid
Glycerol/ethanol solution for SDS-PAGE stains	25% ethanol, 5% glycerin
Guanidinium buffer	Guanidinium HCl 6 M, NaH_2PO_4 100 mM, Tris HCl 10 mM, pH 8
I-1120	Amino acid sequence: Boc-Val-Pro-Arg-AMC, MW: 627.74 g/mol 5 mM stock solution, dissolved in DMSO, storage at -20°C, protected from light (Bachem, Weil am Rhein, Germany)
I-1395	Amino acid sequence: Suc-Leu-Leu-Val-Tyr-AMC, MW: 763.89 g/mol 5 mM stock solution, dissolved in DMSO, storage at -20°C, protected from light (Bachem, Weil am Rhein, Germany)
I-1400	Amino acid sequence: Boc-Phe-Ser-Arg-AMC, MW: 665.75 g/mol 5 mM stock solution, dissolved in DMSO, storage at -20°C, protected from light (Bachem, Weil am Rhein, Germany)
I-1595	Amino acid sequence: Boc-Gln-Gly-Arg-AMC HCl, MW: 653.14 g/mol 5 mM stock solution, dissolved in acetic acid, storage at -20°C, protected from light (Bachem, Weil am Rhein, Germany)
IPTG	1M stock solution; MW 238.3 g/mol, storage at -20°C, protected from light (Biomol, Hamburg, Germany)
Kanamycin	43 mM stock solution containing 25 mg/mL; MW 582.58 g/mol, storage at -20°C
LB medium (Luria-Bertani Medium)	1 liter contains Bacto-Tryptone 10 g, Bacto-Yeast Extract 5 g, NaCl 10 g; dissolved in deionized water, pH adjusted to 7.0 with 5 M NaOH, autoclaved (Bacto-Tryptone and Bacto-Yeast Extract by BD, USA)
Nae I	Restriction endonuclease; recognizes the sequence GCC↓GGC and generates fragments with blunt ends (Roche, Germany)
PCR primers	Based on the known sequences of the KLK (synthesized by Metabion International AG, Munich)
Pfu Ultra II	Fusion HS DNA polymerase (Stratagene, Europe)
PfuUltra® high-fidelity (HF) DNA polymerase	Taken from the QuikChange® II Site-Directed mutagenesis Kit, Catalog #200523 and #200524, storage at -20°C (Stratagene, LaJolla, USA)
Protein marker	Protein marker I 27_1010 (peqlab Biotechnology GmbH, Germany); Coomassie-stained PAA-gels: 10 µL used (correlate to 2 µg protein), Silver-stained PAA-gels: 1 µL used (correlate to 200 ng protein)
Reducing solution for Silver-staining	0.15 mg/mL Farmer's Reducer, freshly prepared before staining
Silver-staining solution	0.1% $AgNO_3$
Storage buffer for	25% ethanol, 5% acetic acid

benzamidine columns	
TE buffer	25 mM Tris HCl, 10 mM EDTA
Transfer Buffer for protein blot	20% ethanol and 50 mM boric acid (lower filters); 5% ethanol and 50 mM boric acid (upper filters), pH 8-9
Urea buffer	Urea 8 M, NaH_2PO_4 100 mM, Tris HCl 10 mM, pH 8
Washing buffer for enterokinase antibodies	0.4 M Tris-HCl, pH 7.4, containing 1 M NaCl and 0.04 M $CaCl_2$, before usage diluted 20 times (Sigma, USA).
Washing-binding buffer for benzamidine columns	50 mM Tris HCl, pH 8, 300 mM NaCl and Tween-20 0.005%, autoclaved, storage at room temperature

The plasmid preparation kits were purchased from Macherey-Nagel, Germany, the restriction enzymes from New England Biolabs, USA and the PCR enzymes from Stratagene, LaJolla, USA. All the cDNA of human tissue kallikreins were isolated by co-workers of the laboratory of the Clinical Research Unit (Department of Gynecology, Technical University of Munich).

IV.1.2 Materials

Amicon Ultra-15 centrifugal filter unit	Device for concentrating <15 mL of protein solution; rinsed with aqua dest. prior to use, centrifuged in Varifuge RF at 2.000x rpm, 4°C for 20 min, concentration checked with Bradford's reagent, rinsed and cleaned with aqua dest. after usage, membrane stored at 4°C with Amicon storing buffer (Millipore, France)
Coulter Mixer	Gently shaking platform
Dialysis bag	Spectra/Por membrane, MWCO: 12-14000, flat width 45 +- 2 mm, diameter: 29 mm. For dialysis, tube with a length of 20 cm was used, it was incubated in nanopure water for 1 h prior to use (Sprectrum Europe B.V., Breda, The Netherlands)
Labcycler	PCR (SensoQuest, Biomedizinische Elektronik GmbH, Göttingen, Germany)
Optiplate 96F	96-well plate, black (Perkin Elmer, Waltham, Massachusetts, USA)
QIAprep Spin Miniprep Kit	(Qiagen, Hilden, Germany)
QuikChange® II Site-Directed mutagenesis Kit	Catalog #200523 and #200524 (Stratagene, LaJolla, USA)
Stirred Ultrafiltration Cell	Cat. No. 5124 (Millipore, France)
Ultrafiltration Membrane	NMWL: 10000, Filter Code: PLGC, Dia: 76 mm, Material: Regenerated Cellulose, Cat. No. PLGC07610 (Millipore, France)

Centrifuges and Equipment

Eppendorf Microcentrifuge, Model 5417R	Maximum capacity: 30 x 1.5–2.0 mL, maximum speed, rpm: 16,400, maximum RCF, g: 25,000, Eppendorf No.: 022621807, temperature control from –9 to 40°C. “Fast Cool” function (Eppendorf, Hamburg, Germany)
Heraeus - Varifuge RF	Heraeus Centrifuge Varifuge RF. 6fold swing-out rotor 2190. Max 5000 rpm. 6 cups 2101. 220 V. 1400 W. -30...+40 °C (Heraeus)
Beckmann Avanti 30	Refrigerated centrifuge, maximum rcf: 15,000 x g, maximum Capacity: 400 mL, operating temperature range: 2 to 40°C, timer: 9 hrs 59 min, pulse or hold (Beckman Coulter, Fullerton, USA)
VICTOR³ 1420	Wallac 1420 Manager, protocol: “josis”, excitation: 355 nm, emission: 460 nm, (Perkin Elmer, Waltham, Massachusetts, USA)
Luminescence Spectrometer LS508	Program: FL Win Lab, (Perkin Elmer, Waltham, Massachusetts, USA), cuvette: quartz, QS 1.000
Semidry Blotter	Biometra (Goettingen, Germany)

IV.2 General molecular biology methods

IV.2.1 Cloning and expression

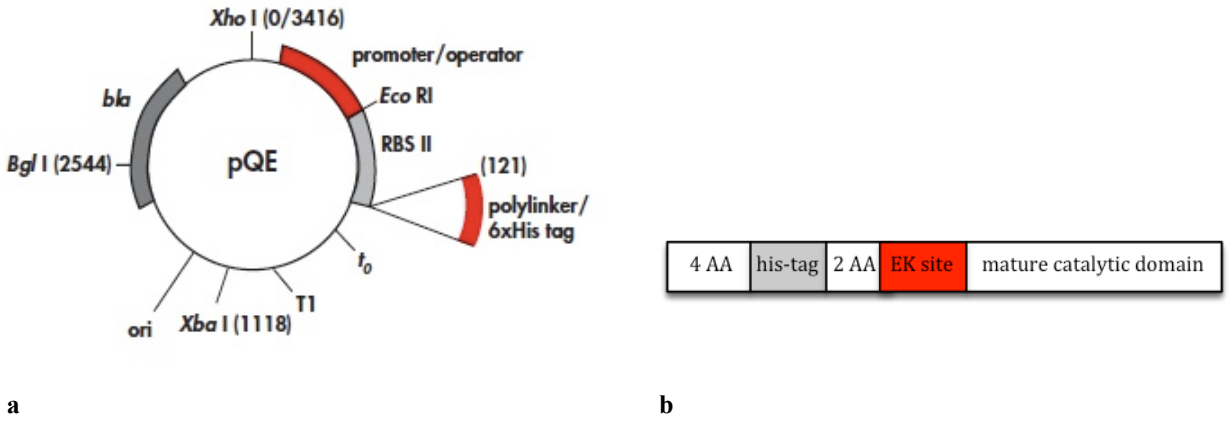
In order to produce pure active KLKs, PCR primers based on the known sequences of the KLK were designed (synthesized by Metabion). At the Clinical Research Unit (Department of Gynecology, Technical University of Munich), RNA was isolated from ovarian and breast cancer tissue or from human keratinocytes and the cDNA sequence encoding the pre-pro-KLK was amplified. Then, the KLK sequence was reduced to its mature domain, which was verified by automated DNA sequencing analysis by Metabion. A short DNA sequence encoding for a his-tag and a restriction site for enterokinase (Asp-Asp-Asp-Asp-Lys↓) was added to the N-terminus of the KLK domain by PCR. The present work continued with the subsequent step, when the construct was cloned into the bacterial expression vector pQE-30 **(fig.IV.1a)**. Finally, a fusion gene was produced **(fig.IV.1b)**, encoding a synthetic pro-form of the KLK protease with an N-terminally located his-tag followed by an enterokinase cleavage site. Expression was regulated with the pREP4 plasmid **(fig.IV.2b)**, which has kanamycin resistance and expresses the repressor protein **(fig.IV.2a)**. By addition of IPTG, which binds to the lac repressor protein, the expression of recombinant proteins encoded by pQE-30 is rapidly induced. After inactivation of the repressor, the RNA polymerase can transcribe downstream from the promotor and the transcript is translated into the recombinant protein. The culture was left to grow at 37°C on a shaking platform (200 rpm) for 16 h.

Transformed cells grew over night at 37°C in liquid culture containing

Bacteria	50 µL	
LB medium	50 mL	
Ampicillin	50 µL	(final concentration 100 µg/mL)
Kanamycin	50 µL	(final concentration 30 µg/mL)

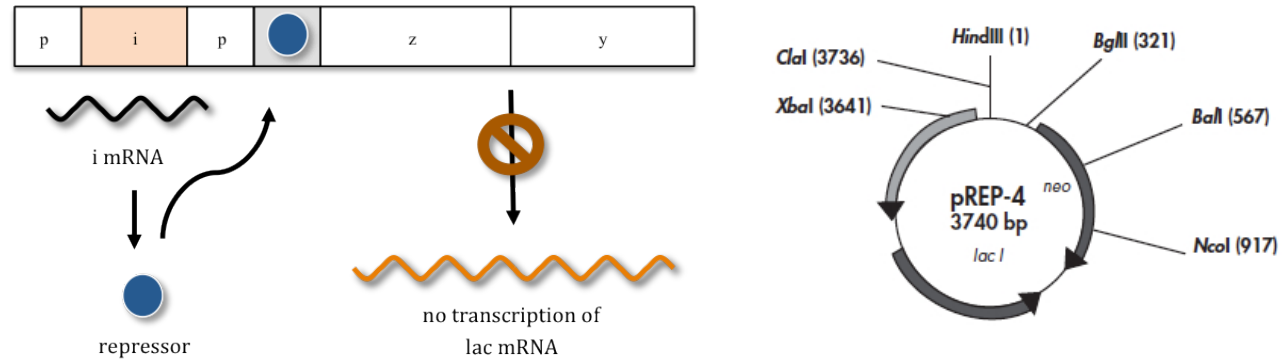
From the overnight culture a 1:100 dilution was inoculated with freshly prepared medium containing

2xTY medium	1 L	
Ampicillin	1 mL	(final concentration100 µg/mL)
Kanamycin	1 mL	(final concentration 30 µg/mL)



a **b**

Figure IV.1 Strategy for the generation of a KLK expression vector. Fig. a depicts the pQE vector containing a strong promoter and a start codon followed by a his tag. The coding region of the protease was amplified and then cloned into the vector. Fig. b shows the construct with mature catalytic domain of the KLK, an appropriate enterokinase cleavage site (EK site) and a his-tag that was generated after cloning the recombinant protein into pQE. Taken and modified from The QIAexpressionist™, 2003.



a **b**

Figure IV.2 Regulation of recombinant KLK expression in *E. coli*. The pREP4 plasmid leads to the overexpression of the lac repressor and ensures complete shut-down of recombinant protein expression in the absence of IPTG. Fig. a depicts the lac operon. The lac repressor binds the operator region and represses transcription from the promotor. With the addition of IPTG the lac repressor dissociates from DNA and induces transcription. P indicates promotor. Fig. b shows the pREP-4 vector that expresses the lac repressor protein. Using the pREP-4 vector the expression of the recombinant proteins was rapidly regulated by adding IPTG. Taken and modified from the QIAexpressionist 06/2003.

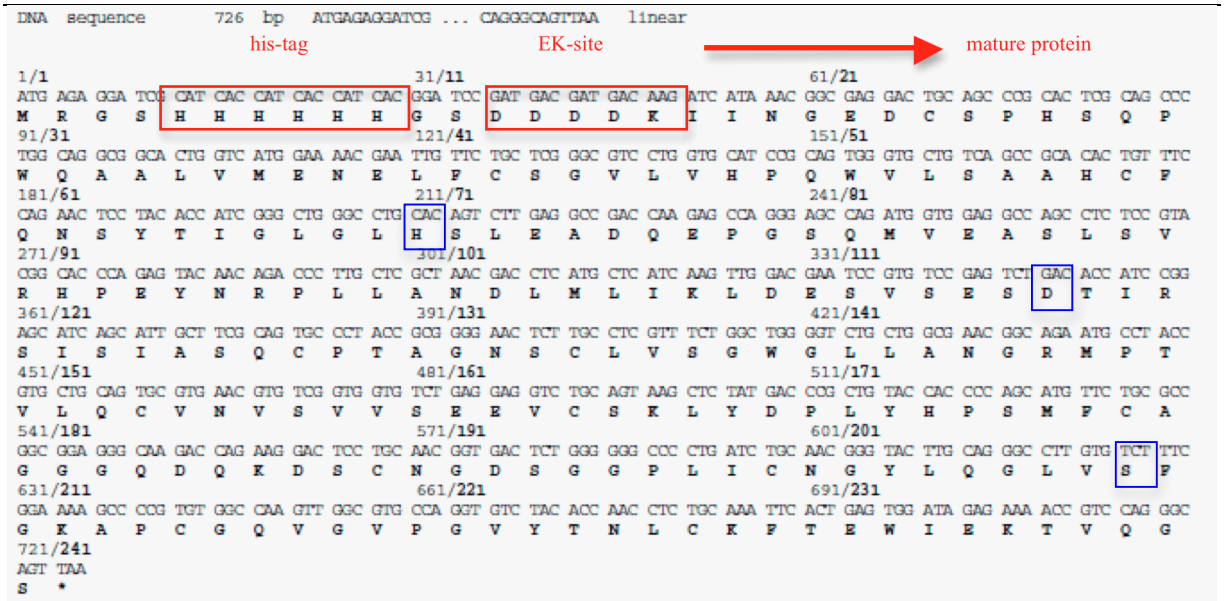
Then, the culture was left to grow at 37°C on a shaking platform (200 rpm) until it reached an OD_{600} reading of 0.6. - 0.8. At the optimal OD_{600}, cell expression was induced by adding IPTG to a final concentration of 1 mM in the culture. After 4 h of induction, cells were harvested and spun at 21,000 rpm at 4°C for 10 min. Subsequently, the mixture was fractionated by centrifugation, yielding a pellet of dense cellular material at the bottom of the centrifuge tube and a supernatant above. Supernatants were discarded and the cell pellets frozen at -80°C for 1 h. The frozen pellets were resuspended in buffer containing 6 M

guanidinium HCl, 100 mM NaH_2PO_4, 10 mM Tris HCl, pH 8 and 10 mM β-mercapthoethanol. The resuspended cells were incubated at room temperature for 2 h under constant stirring. The cellular debris was then removed by centrifugation at 21,000 rpm and 4°C for 5 min. At this stage, the raw lysate contained the recombinant protein among several other proteins derived from the bacteria. The recombinant protein, thus, was purified by Ni^{2+} - NTA affinity chromatography.

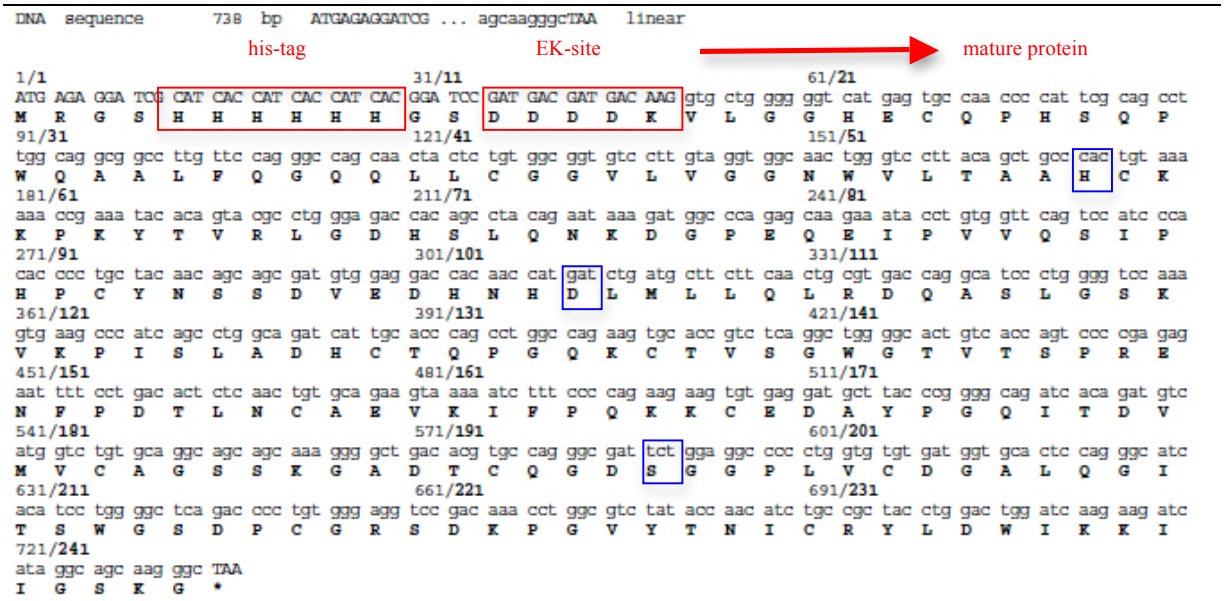
IV.2.1.1 DNA sequences and primers

Following **figures IV.3 a-e** display the DNA sequences of each KLK4, 8, 15 fl, 15 ntfl and 15 S/A. Red boxes indicate the his-tag or EK-site, blue boxes the catalytic triad. Black line **(fig.IV.3 d)** stands for the amino acid sequence lacking in KLK15 ntfl compared to KLK15 fl. Green box **(fig.IV.3 e)** shows the mutated alanine.

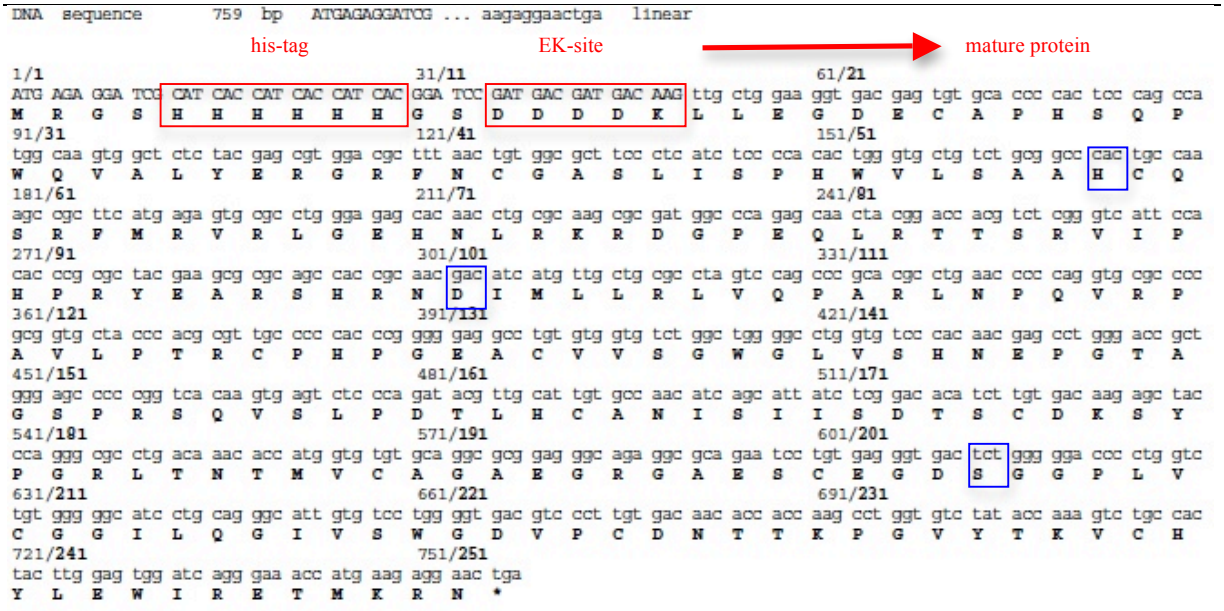
a: KLK4



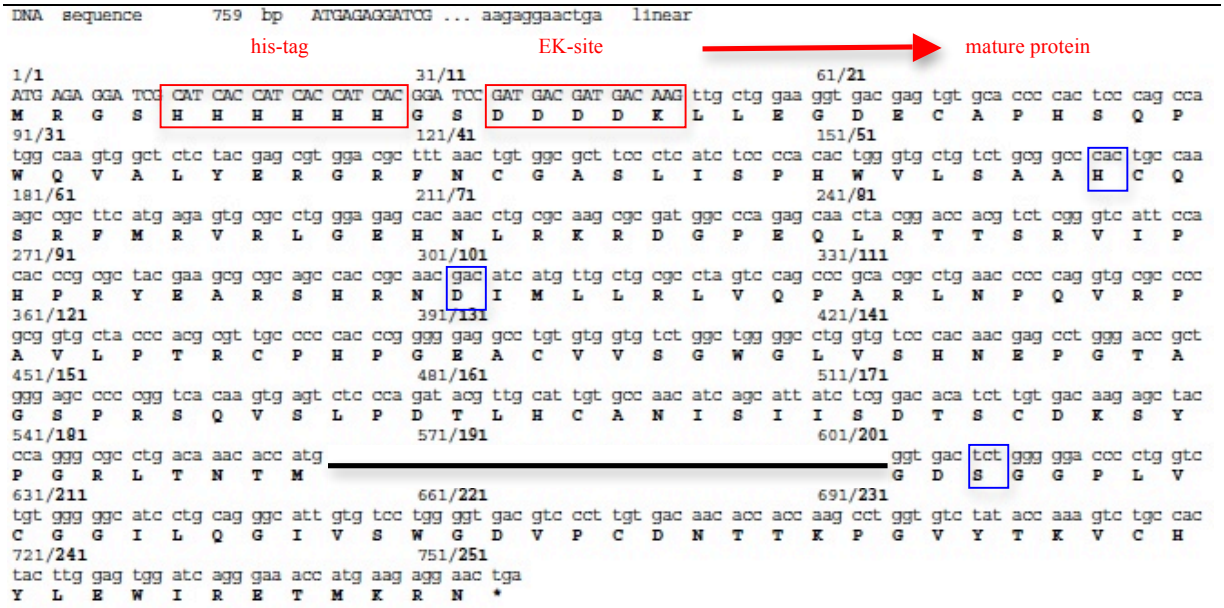
b: KLK8



c: KLK15 fl



d: KLK15 ntfl



e: KLK15 S/A

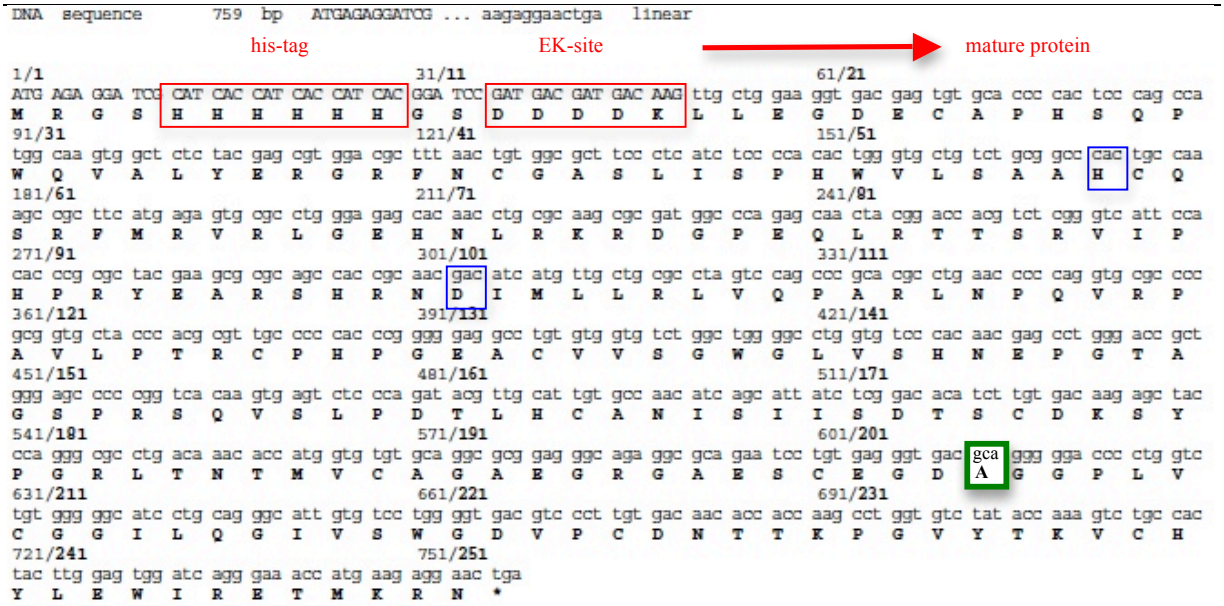


Figure IV.3 Amino acid sequence of the synthetic pro-forms of KLK4, KLK 8, KLK15 fl, KLK15 ntfl and KLK15 S/A. Red boxes indicate the his-tag or EK-site, blue boxes the catalytic triad. The black line (fig.IV.3 d) stands for the amino acid sequence lacking in KLK15 ntfl compared to KLK15 fl. The green box (fig.IV.3 e) shows the mutated alanine.

IV.2.2 Site-directed mutagenesis

Mature KLKs contain a highly conserved catalytic triad of histidine (H), aspartic acid (D) and serine (S) (Yousef et al., 2001). In order to further analyze the structure, function and activity of KLK15, a mutant of the enzyme lacking enzymatic activity was produced. For *in vitro* mutagenesis, two forward and reverse primers were designed (Metabion, **fig.IV.4**) with the codon to replace serine position with alanine. Both primers ended with G/C bases and they included a restriction site for Nae I (Roche, Germany), a restriction enzyme that recognizes the sequence GCC↓GGC and generates fragments with blunt ends. Shortly before use, the lyophilized primers were dissolved in RNase/DNase-free water. To extract the pQE-30 plasmid encoding for KLK15 fl, a mini preparation was performed using the QIAprep Spin Miniprep Kit **(fig.IV.5)**. The yield was evaluated using a photometer. The pure plasmid DNA displayed a final concentration of 0.05 µg/µL. The QuikChange® II Site-Directed mutagenesis Kit (Stratagene, LaJolla, USA, **fig.IV.6**) allows site-specific mutation in a double-stranded plasmid. In order to generate the S/A strand thermal cycling was performed. For PCR thin-walled tubes were utilized. The super-coiled double-stranded vector DNA (dsDNA) with the insert of interest was denatured. This step was followed by annealing of the oligonucleotide primers at 67°C, each complementary to opposite strands of the vector. Subsequently the primers were extended during temperature cycling by PfuUltra HF DNA polymerase, without primer displacement to generate a mutated plasmid containing staggered nicks. Subsequently the product was treated with Dpn I. The Dpn I endonuclease (target sequence: 5′-Gm_6ATC-3′) is specific for methylated and hemi-methylated DNA and was used to digest the parental DNA template to select for the mutation containing synthesized DNA. This is due to the fact that DNA isolated from almost all *E. coli* strains is highly methylated and for this reason susceptible to Dpn I digestion. Finally, the nicked plasmid DNA with the desired serine to alanine mutation was transformed into XL1-Blue competent cells.

KLK15-S/A-F 5’ GTG AGG GTG ACG CCG GCG GAC CCC TGG TC
KLK15-S/A-R 5’ GAC CAG GGG TCC GCC GGC GTC ACC ATC AC

Figure IV.4 Generation of KLK15 S/A. Sequence of forward and reverse primer generated in order to replace serine with alanine by *in vitro* mutagenesis. Primers were synthesized by Metabion, Germany.

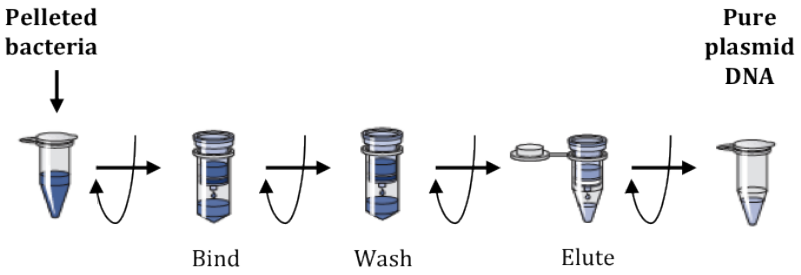


Figure IV.5 Extraction of plasmid DNA from bacteria. After bacteria are lysed, both proteins and chromosomal DNA are precipitated. DNA binds to the silica membrane, the solution is washed, removing unwanted proteins. When resuspended, the pure plasmid DNA renatures and can be extracted this way. From the Qiagen QIAprep Miniprep Handbook, QIAexpressionist 12/2006.

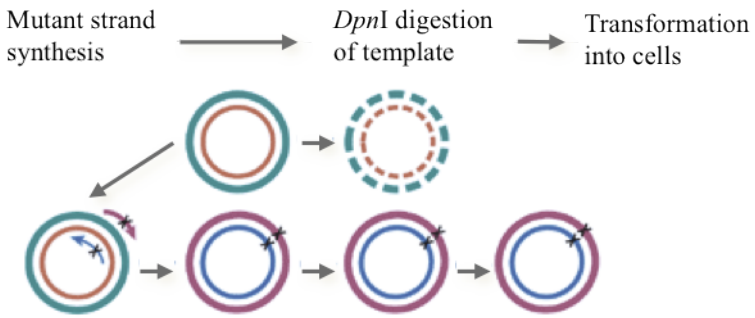


Figure IV.6 The site-directed mutagenesis method. For mutant strand synthesis, thermal cycling is performed: the DNA template is denatured, followed by annealing of the mutagenic primers containing the desired mutation, to finally extend the primers with PfuUltra DNA polymerase. As a next step, the parental methylated and hemimethylated DNA is digested with Dpn I. Finally, the mutated molecule is transformed into competent cells for nick repair. From the QuikChange II manual (Stratagene, LaJolla, USA).

The samples for mutant strand synthesis reaction (thermal cycling) for KLK15 S/A pQE-30 were prepared as indicated below:

dH_2O	36 µL
10x reaction buffer	5 µL
Forward primer	1 µL
Reverse primer	1 µL
dNTPs mix	5 µL (from a solution containing 2.5 mM of each dNTP)
Plasmid pQE-30	1 µL
PfuUltra DNA polymerase	1 µL (2.5 U/µL)

Following cycling parameters were applied:

Cycle	Temperature	Time
1.	95°C	2-3 min
2.	95°C	20 sec
3.	67°C	20 sec
4.	72°C	90 sec
5.	Step 2.–4.	18 times
6.	72°C	3 min
7.	4°C	∞

For PCR, the labcycler SensoQuest was used (Biomedizinische Elektronik GmbH, Göttingen, Germany).

Thereafter, the amplified product was digested by adding 1 µL of the Dpn I restriction enzyme (10 U/µL) directly to the amplification reaction. The sample was gently and thoroughly mixed by pipetting the solution up and down several times. Subsequently, the reaction mixture was incubated at 37°C for 1 h to digest the parental (i.e., the non-mutated) super-coiled dsDNA. Finally, the sample was stored at -20°C over night.

The samples for Dpn I digestion of the amplification product of KLK15 S/A pQE-30 were prepared as indicated below:

PCR reaction sample	
Buffer 4	4 µL
Dpn I	1 µL (10 U/µL)

To transform the new plasmid into XL1-Blue competent cells, Eppendorf tubes were cooled down on ice after filling them with 20 µL TE buffer. The samples from the Dpn I digestion were thawed on ice and vortexed. Furthermore, 100 µL of XL1-Blue competent cells were gently defrosted on ice for 15 min but not mixed or vortexed. Subsequently 5 µL of Dpn I digested plasmids were added and incubated on ice for 30 min. As a next step the transformation reaction was heat-pulsed for 1 min and 45 sec at 37°C and then placed on ice for 5 min. Thereafter, 1 mL of 2xTY broth, pre-heated to 37°C, was added to the sample and gently mixed by inverting the tube several times. Afterwards, the reaction was incubated at 37°C for 1 h. Following the incubation step, cells were spun down at 5000 rpm (Eppendorf Microcentrifuge, Model 5417R) for 1 min at room temperature, supernatant was discarded, and cells were resuspended in the minimal amount of media that was left in the tube. Then the sample was plated on LB-agar plate containing ampicillin (100 µg/mL), the appropriate antibiotic for selection of the plasmid. The plated cells with the mutated plasmid were incubated at 37°C over night. In order to pre-select candidates of the site-directed

mutagenesis of KLK15 S/A, a control digestion was performed with plasmid DNA of four clones, picked beforehand, utilizing the built-in novel restriction site for Nae I and an existing one for EcoR I **(fig.IV.7)**. Bacteria were grown in 5 mL LB broth containing 5 µL ampicillin (final concentration 100 µg/mL) at 37°C on a shaking platform (200 rpm) for 16 h. DNA was isolated utilizing the QIAprep Spin Miniprep Kit for digestion with the restriction enzymes Nae I and EcoR I. The results were checked by electrophoresis of 10 µL of the product with 2.5 µL 5x DNA loading dye on a 1% agarose gel.

The master mix for control digestion with Nae I plus EcoR I of the KLK15 S/A pQE-30 plasmid was prepared as indicated below:

ddH_2O	18 µL
Buffer 1	10 µL (10x)
Nae I	8 µL (10 U/µL)
EcoR I	4 µL (20 U/µL)

For each clone, 6 µL of the DNA sample was added to 4 µL master mix (ddH_2O 24 µL, buffer 1 10x 10 µL, Nae I 10 U/µL 6 µL) and incubated at 37°C for 1 h. The result was checked by electrophoresis of 10 µL of the product and 2.5 µL of 5xDNA loading dye, stained by ethidium bromide on a 1% agarose gel that was run at 90 V. Finally, the mutated plasmid was sequenced by Metabion, Germany to confirm the results. To express the mutated KLK15 S/A, pQE-30 was transformed into the M15 *E. coli* strain harboring the pREP plasmid. For this 0.5 µL DNA was extracted from each clone 1 to 4 and added to 30 µL transformation-competent M15 cells and 5 µL TE buffer. Hereafter, the samples were incubated on ice for 30 min, the transformation reaction was heat-pulsed for 1 min and 45 sec at 37°C and placed on ice for 5 min. Subsequently, 1 mL of 2xTY broth, pre-heated to 37°C, was added to each sample and gently mixed by inverting the tube several times. Afterwards, the reaction was incubated at 37°C for 1 h, then cells were spun down at 5000 rpm (Eppendorf Microcentrifuge, Model 5417R) for 1 min at room temperature. The pelleted cells were resuspended in 5 mL LB medium with 5 µL ampcillin (from a 270 mM stock solution) and 5 µL kanamycin (from 43 mM stock solution) and grown over night at 37°C on a shaking platform.

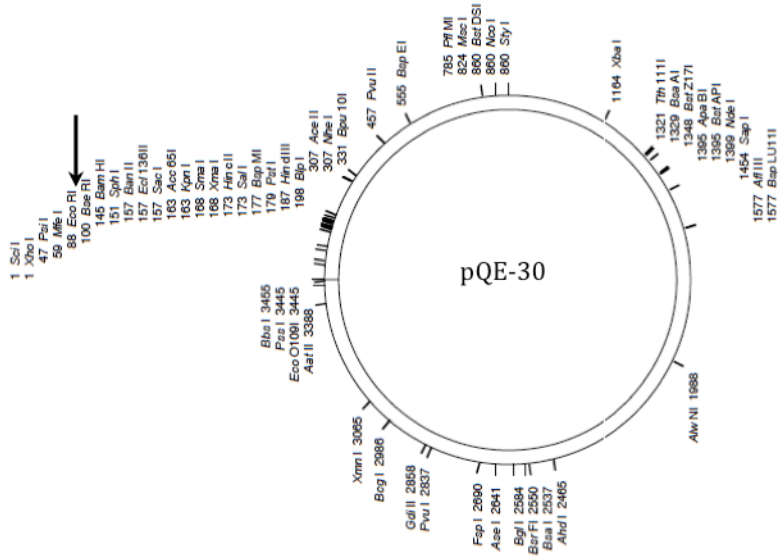


Figure IV.7 The pQE-30 plasmid. The unique restriction sites are indicated. The arrow indicates the restriction site for EcoR I.

The next day, 2x 300 µL of each clone was added to 2x 3 mL of 2xTY medium with 3 µL ampcillin (from a 270 mM stock solution) and 3 µL kanamycin (from 43 mM stock solution) and incubated at 37°C on a shaking platform (200 rpm) until it reached an OD_{600} reading of 0.6. - 0.8. Subsequently, cell expression was induced by adding IPTG to a final concentration of 1 mM in the culture. After 3.5 h of induction, cells were harvested and spun at 15,000 rpm at room temperature for 2 min. The supernatant was discarded and the cell pellets frozen at -80°C for 20 min. The bacteria were lysed under strong denaturing conditions to extract protein from inclusion bodies by utilizing cracking buffer containing 1% 2-mercaptoethanol, 1% sodium dodecyl sulfate and urea buffer. In order to visualize the proteins by SDS-PAGE, 40 µL cracking buffer, 20 µL 3x reducing loading dye was added to each pellet and heated at 95°C for 3 min. Hereafter, 20 µL of each sample was run on a gel and the bands were visualized by Coomassie staining. The clone that appeared to have the highest expression yield of KLK15 S/A was chosen for further production and purification of the mutated protein.

IV.3 Protein handling

IV.3.1 Protein analysis

Proteins play an important role in a variety of biological processes such as catalysis, signal transmission, and structural support. Each protein has different structure levels, from primary to tertiary, even quarternary structure and it is folded into a distinctive three-dimensional structure, allowing it to interact with different molecules and perform its specialized function. Typical steps in the investigation of the function of a protein are isolation and purification. Different proteins can be distinguished from one another by their size, charge, binding affinity and solubility. After purification, the amino acid sequence can be determined for further analysis. Monoclonal antibodies can detect proteins *in vitro* and *in vivo* and, therefore, help to provide more information on their physiological context. Additional studies on the protein may involve nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography may be used to study the three-dimensional structure (Stryer et al., 2002).

IV.3.1.1 SDS polyacrylamide gel electrophoresis

To determine whether the purification was effective, it is crucial to visualize the protein. This is possible with SDS polyacrylamide gel electrophoresis, which allows protein separation according to molecular mass.

IV.3.1.1.1 12% polyacrylamide gel

Polymerized acrylamide forms a cross-linked polymer network with pores of different sizes, related to the amount of acrylamide used. For example, gels with 7% acrylamide have larger pores than 12% gels, therefore, they can be used to resolve large proteins. Polymerization of acrylamide is catalyzed in the presence of TEMED and APS. The gel consists of two gels (**fig.IV.8**): the separating gel and the stacking gel. The latter is poured over the top of the separating gel, it has a lower concentration of acrylamide, lower pH and a different ionic content. It allows the proteins to be concentrated in a tight band before entering the separating gel.

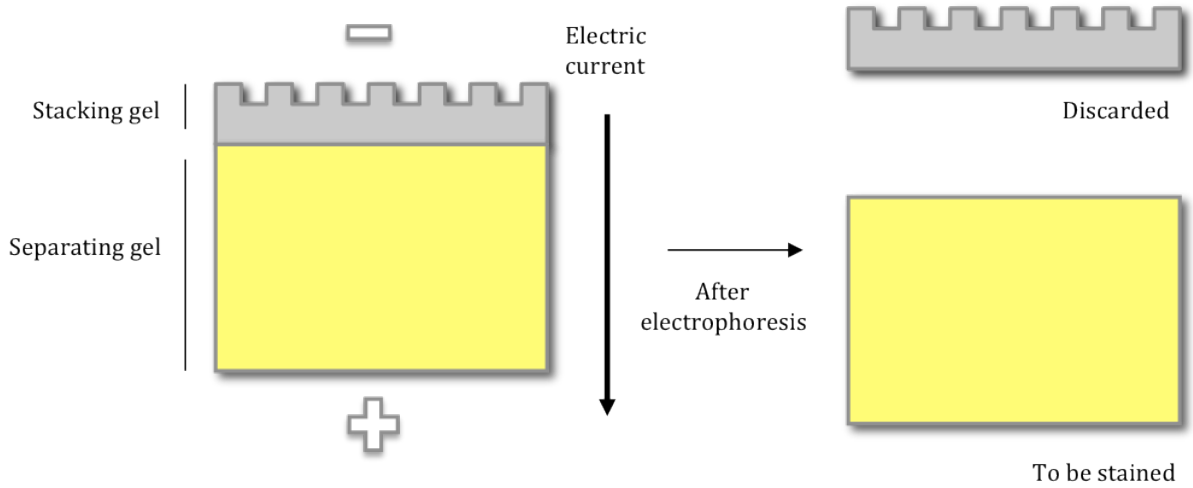


Figure IV.8 The gel consists of the stacking and the separating gels. The stacking gel contains the protein sample, its concentration of acrylamide and pH are both lower. Moreover, it has a different ionic content. In this way it allows the proteins to be concentrated in a tight band before entering the separating gel. Afterwards the separating gel is stained in order to visualize the proteins.

To prepare two separating gels, 12% polyacrylamide, 1 mm diameter

Water ultrapure	4.3 mL
1.5 M Tris pH 8.8	2.5 mL
10% SDS solution	100 µL
40% Acrylamide-Bisacrylamide 29:1	3 mL
10% APS	80 µL
TEMED	8 µL

To prepare stacking gels, 1 mm diameter

Water ultrapure	17.2 mL
0.5 M Tris pH 6.8	7.2 mL
10% SDS solution	280 µL
40% Acrlyamide-Bisacrylamide 29:1	3.6 mL
to be added before polymerization:	
10% APS	80 µL
TEMED	8 µL

IV.3.1.1.2 Electrophoresis

Protein samples are dissolved in a solution with sodium dodecyl sulfate (SDS), an anionic detergent that disrupts noncovalent interactions in native proteins, which results in unfolding of the protein. SDS binds amino acid residues and builds up complexed with the protein. In order to reduce disulfide bonds, 2-mercaptoethanol (2-thioethanol) or dithiothreitol is added. A thin, vertical slab of polyacrylamide gel is used for electrophoresis. Gels serve as a molecular sieve that enhances separation. Typically, for a separation on the basis of mass a polyacrylamide gel and denaturing conditions are used. The net negative charge is proportional to the mass of the protein (**fig.IV.10**). Upon application of the electric current, negative charged proteins migrate towards the anode (**fig.IV.9**). Small molecules move much more rapidly through the gel pores in comparison with large molecules that are hindered more. After electrophoretic separation, the protein in the gel can be visualized by staining with silver or a dye such as Coomassie. The mobility of a protein in the gel is proportional to its mass; large molecules migrate through the gel at a slower rate than small molecules. SDS-polyacrylamide gel electrophoresis is a rapid and sensitive method for detection of small amounts of proteins. Coomassie staining can detect as little as 0.1 μg of protein, while silver staining can detect 0.02 μg (Stryer et al., 2002).

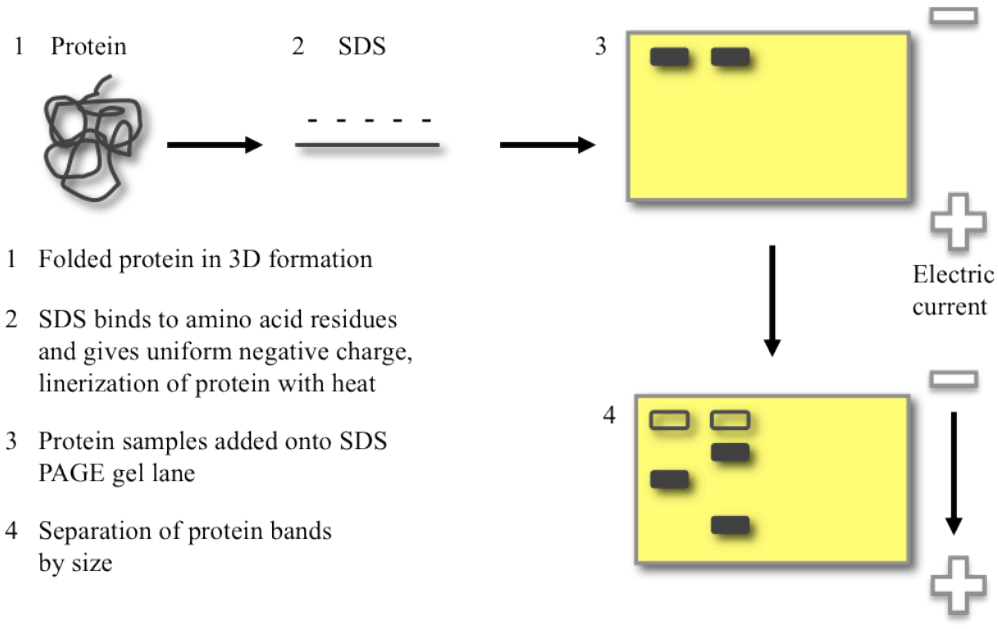


Figure IV.9 Principle of gel electrophoresis. Proteins are dissolved in SDS, disrupting noncovalent interactions and therefore resulting in unfolding. Afterwards, the protein is linearized by heating. The samples are then added to the SDS PAGE. Upon application of the electric current, negative charged proteins migrate towards the anode. Small molecules move faster through the gel pores in comparison with large molecules that are hindered more. After electrophoretic separation, the protein in the gel can be visualized by staining it with silver or a dye such as Coomassie.

$$v = \frac{Ez}{f}$$

Figure IV.10 Formula displaying the velocity of migration *(v)* of the protein. It depends on the electric field strength *(Ez)* and the frictional coefficient *(f)*, so that the net negative charge is proportional to the mass of the protein. This explains how proteins of different masses and charges will move differently in a gel after application of an electric current.

IV.3.1.1.3 Coomassie staining

The gel was incubated in fixation solution (45% ethanol, 10% acetic acid) for at least 20 min on a shaker with gentle agitation and then washed in deionized water. As a following step, the gel was incubated for 40 min with a solution containing 0.1% Coomassie in 10% acetic acid, then in deionized water. It was destained with 10% acetic acid and dried for 30 min in glycerol/ethanol solution.

IV.3.1.1.4 Silver staining

The gel was incubated in fixation solution for at least 20 min on a shaker with gentle agitation, then washed 2 x 5 min in deionized water. Reducing solution (20 mL per gel) was added and incubated for 5 min. The gel was washed 5 times for 10 min each in deionized water. As a following step, it was incubated with 0.1% $AgNO_3$ for 30 min. Afterwards, the gel was washed quickly for 45 sec, and then developing solution was added. To stop developing 10% acetic acid was added in excess. The gel was then dried for 30 min in glycerol/ethanol solution.

IV.3.1.2 Affinity chromatography

Protein purification relies on exploiting as many of the physical properties of the protein as are necessary for separation from all other proteins. Most commonly, these steps include methods that separate by molecular weight, charge, hydrophobicity, absorptive properties or group-specific interactions. One of the most efficient methods is affinity chromatography. This technique takes advantage of the high affinity of certain proteins for specific chemical groups. It can be used either to purify specific proteins or to remove unwanted proteins from a solution. For example, benzamidine sepharose binds the active center of trypsin-like serine

proteases and therefore allows their extraction from a solution. Affinity chromatography is most effective when the interaction of the protein and the molecule that is used is highly specific (Beynon et al., 2001). The polyhistidine-tag was used for affinity purification of the recombinant proteins.

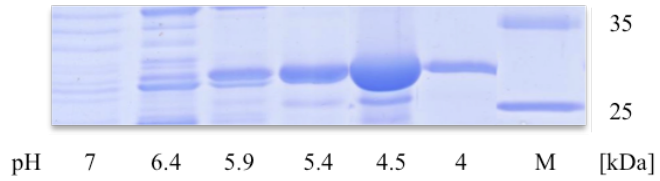


Figure IV.11 KLK15 fl purification by affinity chromatography using Ni^{2+}-NTA -agarose. At pH 8.0 the his-tagged protein binds to the affinity matrix, from pH 7.0 to 5.9 the molecule/matrix complex is washed in order to remove bacterial proteins. Finally, from pH 5.4 to 4.0 the recombinant protein is liberated from the matrix and the solution appears pure. Proteins are separated by 12% SDS-PAGE and subsequently Coomassie-stained.

The raw lysate containing the recombinant KLK, among other proteins derived from the bacteria, was incubated with Ni^{2+}-NTA-agarose. This affinity media contains nickel as a bound metal ion to which the polyhistidine-tag binds with micromolar affinity. High levels of expression of recombinant proteins can lead to a formation of insoluble aggregates, such as inclusion bodies that are often found in *E. coli*. Therefore, a strong denaturant such as 8 M urea is necessary to completely solubilize inclusion bodies and his-tagged proteins. The nickel-nitrilotriacetic acid sepharose column (Qiagen) was equilibrated with urea buffer (urea 8 M, NaH_2PO_4 100 mM, Tris HCl 10 mM, pH 8). The supernatants containing the KLK constructs with a his-tag and an enterokinase cleavage site were then applied to the NTA-agarose. The column was then washed extensively using the same urea buffer but with decreasing pH (8.0 – 6.0) to remove proteins that do not specifically interact with the nickel ion. At a pH of approximately 6.0 the histidine residues become protonated. Therefore, the his-tagged protein will no longer be able to bind to the nickel ions and will dissociate from the NTA-agarose. The purity and amount of protein was assessed by SDS-PAGE **(fig.IV.11)**. The resulting solutions with the KLK were then stored at 4°C for further analysis and refolding (Qiagen, The QIAexpressionist™, 2003).

IV.3.1.3 Protein structure and function

Proteins play an important role in essentially all biological processes. They consist of amino acids that are arranged in a linear chain and then joined together by peptide bonds between the carboxyl and amino groups of the amino acid residues. During transcription of a gene, the primary transcript can be processed using various forms of post-transcriptional modification. As a following step the mRNA signal is translated into an amino acid sequence encoding for the protein. Prokaryotes translate the mRNA in the cytosol using ribosomes. A 30S ribosomal subunit, formylmethionyl-$tRNA_f$ (the special initiator tRNA that recognizes AUG) and mRNA build a complex which is then joined by a 50S ribosomal subunit forming a 70S initiation complex. Eukaryotes produce their mRNA in the cell nucleus. The mRNA is transported to the cytosol, which is then translated into protein by an 80S ribosome complex. Moreover, eukaryotes can modify the translated protein in cell organelles such as the endoplasmic reticulum or the Golgi apparatus, which gives the protein a distinct tertiary or three-dimensional structure (Stryer et al., 2002). The primary structure is the amino acid sequence held together by peptide bonds. Secondary structure refers to regular sub-structures, such as alpha helix and beta sheet. The tertiary structure is the three dimensional structure of a protein. The quaternary structure consists of a complex of protein molecules.

IV.3.1.3.1 Protein refolding

High expression leads to insoluble inclusion bodies. With agents such as urea or guanidine hydrochloride the noncovalent bonds are effectively disrupted. Moreover, by using reducing reagents such as 2-mercaptoethanol the disulfide bonds are reversibly opened so that the disulfides (cystines) are fully converted to sulfhydryls (cysteines). In order to reduce and denature proteins they are treated with 2-mercaptoethanol in 8 M urea, generating a randomly coiled polypeptide chain lacking cross-links and enzymatic activity (**fig.IV.12**). Thus, it was essential to refold the purified KLKs derived from bacteria. In our protein refolding protocols (**tables IV.1 a-d**), which were elaborated by Dr. Mekdes Debela (Max-Planck-Institute Munich, Germany), indications mentioned in the Qiagen Manual (QIAexpressionist 06/2003) were taken into consideration. In order to avoid precipitation of the protein a low protein concentration (about 10–50 μg/mL) was utilized in refolding. The use of thiol reagents contributed to a correct formation of disulfide bonds. To create the necessary oxidizing potential and in order to form and break disulfide bonds in folding intermediates a redox pair

of reduced glutathione (GSH) and oxidized glutathione (GSSG) was required. During refolding, denatured proteins were formed and had to be removed by dilution or dialysis. Glycin, which displays remarkable solubilization properties, was used in order to stabilize the protein upon refolding. In addition other stabilizers such as glycerol (5–20%) and ethylene glycol, as well as glucose and sucrose (10%) were helpful during the refolding process, since they are known to act by stabilizing hydrophobic interactions. However, these co-solvants can also stabilize aggregate formation of the protein. Furthermore, salts such as KCl, NaCl and $MgCl_2$ were included. After the elution of the denatured purified KLK, the protein was incubated with 10 mM DTT over night at 4°C on a magnetic stirrer. For dialysis a Spectra/Por membrane was used (MWCO: 12 -14,000) as a dialysis bag. All refolding methods that were applied in our laboratory were at 4°C on a magnetic stirrer at pH 8.0. The protein was dialyzed against a dialysis buffer containing 4 M urea Tris HCl and NaCl over night at 4°C. To refold the construct the high salt concentration was step-wise reduced. The next day the dialysis bag containing the KLK was put into refolding buffer 1 for 12 h, and then the buffer was exchanged with refolding buffer 2, where the KLK was dialyzed for 48 h. To terminate the refolding process and to provide an appropriate buffer for storing, the refolded KLK was dialyzed against a storage buffer for 12 h containing 100 mM NaCl, 50 mM Tris HCl and 0.005 % Tween-20. In order to completely remove all reducing and oxidizing reagents, the storage buffer was changed to a freshly prepared one after 4 h. Finally, the refolded protein was aliquoted and stored at -80°C for further analysis.

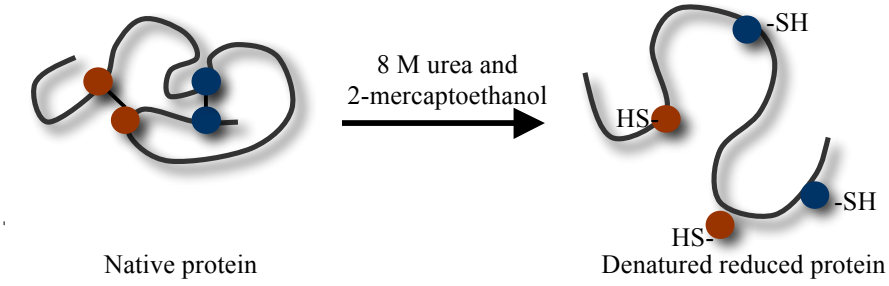


Figure IV.12 Effects of urea and 2-mercaptoethanol. Urea disrupts the noncovalent bonds of proteins and reversibly cleave the disulfide. The product is a randomly coiled polypeptide chain lacking cross-links. This property is used during the refolding process of proteins in order to denature them.

Refolding protocols for KLK proteases:

KLK	Dialysis buffer	Refolding buffer		Storage buffer
a				
KLK4	urea 4 M NaCl 100 mM Tris HCl 50 mM Tween-20 0.005 % pH 8.0 At 4°C over night	(1) urea 2 M NaCl 100 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2.2 mM glutathione red. 5 mM glutathione ox. 0.5 mM Tween-20 0.005 % pH 8.0 At 4°C over night	(2) urea 1 M NaCl 100 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2.2 mM glutathione red. 5 mM glutathione ox. 0.5 mM Tween-20 0.005 % pH 8.0 At 4°C for 48 h	NaCl 100 mM Tris HCl 50 mM Tween-20 0.005 % pH 8.0 At 4°C for 4 h, exchange to a fresh storage buffer, dialysis over night

KLK	Dialysis buffer	Refolding buffer		Storage buffer
b				
KLK8	urea 4 M NaCl 350 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2 mM $CaCl_2$ 2.2 mM $ZnCl_2$ 50 µM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % pH 8.0 At 4°C over night	(1) urea 2 M NaCl 350 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2 mM $CaCl_2$ 2.2 mM $ZnCl_2$ 50 µM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % pH 8.0 At 4°C over night	(2) urea 1 M NaCl 350 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2 mM $CaCl_2$ 2.2 mM $ZnCl_2$ 50 µM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % pH 8.0 At 4°C for 48 h	NaCl 100 mM Tris HCl 50 mM Tween-20 0.005 % pH 8.0 At 4°C for 4 h, exchange to a fresh storage buffer, dialysis over night

KLK	Dialysis buffer	Refolding buffer		Storage buffer
c				
KLK15 fl, **KLK15** **ntfl,** **KLK15** **S/A:** Protocol I	urea 4 M NaCl 350 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2 mM $CaCl_2$ 2.2 mM $ZnCl_2$ 50 µM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % pH 8.0 At 4°C over night	(1) urea 2 M NaCl 350 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2 mM $CaCl_2$ 2.2 mM $ZnCl_2$ 50 µM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % pH 8.0 At 4°C over night	(2) urea 1 M NaCl 350 mM Tris HCl 50 mM glycine 50 mM $MgCl_2$ 2 mM $CaCl_2$ 2.2 mM $ZnCl_2$ 50 µM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % pH 8.0 At 4°C for 48 h	NaCl 100 mM Tris HCl 50 mM Tween-20 0.005 % pH 8.0 At 4°C for 4 h, exchange to a fresh storage buffer, dialysis over night

KLK	Dialysis buffer	Refolding buffer		Storage buffer
d				
KLK15 fl,	urea 4 M	(1)	(2)	NaCl 100 mM
KLK15	NaCl 100 mM	urea 2 M	urea 1 M	Tris HCl 50 mM
ntfl,	Tris HCl 50 mM	NaCl 350 mM	NaCl 350 mM	Tween-20 0.005 %
KLK15	Tween-20 0.005 %	Tris HCl 50 mM	Tris HCl 50 mM	pH 8.0
S/A:	pH 8.0	glycine 50 mM	glycine 50 mM	
Protocol II	At 4°C over night	Sucrose 50 mM	Sucrose 50 mM	At 4°C for 4 h,
		$CaCl_2$ 2.2 mM	$CaCl_2$ 2.2 mM	exchange to a fresh
		KCl 2.2 mM	KCl 2.2 mM	storage buffer, dialysis
		L-arginine 500 mM	L-arginine 500 mM	over night
		glutathione red. 5 mM	glutathione red. 5 mM	
		glutathione ox. 0.5 mM	glutathione ox. 0.5 mM	
		Tween-20 0.005 %	Tween-20 0.005 %	
		PEG3350 0.055%	PEG3350 0.055%	
		pH 8.0	pH 8.0	
		At 4°C over night	At 4°C for 48 h	

Tables IV.1 Refolding protocols for KLK proteases. To improve the yield of KLK15 different protocols were applied. a: KLK4, b: KLK8, c: KLK15 fl, ntfl and S/A Protocol I; c: KLK15 fl, ntfl and S/A Protocol II.

IV.3.1.3.2 Drop-wise method

Additionally, the so-called drop-wise method was employed, which is known to be a more effective way of refolding certain proteases (protocol **table IV.2**). After eluting the denatured purified KLK, the protein was incubated in a buffer with 40 mM DTT for 8 h at room temperature. All refolding methods that were applied in our lab were at 4°C on a magnetic stirrer. The protein was dialyzed using a Spectra/Por Membrane (MWCO: 12 -14,000) against a dialysis buffer containing 4 M urea HEPES and NaCl with pH 7.5 over night at 4°C. Thereafter, the dialysis bag containing the KLK was placed in refolding buffer (1) over night. Subsequently, the solution with the construct was carefully dropped from a height of 50 cm into a vial containing 350 mL of refolding buffer (2) on a magnetic stirrer. The KLK was then incubated in this buffer for 24 h. Finally, the protein solution was concentrated to the desired volume using a Stirred Ultrafiltration Cell (Cat. No. 5124 by Millipore) with an Ultrafiltration Membrane (MWCO: 10,000, by Millipore). Additives and salt solution were dialyzed against a storage buffer for 4 h at 4°C, then the storage buffer was exchanged to a freshly prepared one. Finally, the protein solutions were aliquoted and stored at -80 ºC, prior to use.

KLK	Dialysis buffer	Refolding buffer		Storage buffer
KLK15 fl, KLK15 ntfl, KLK15 S/A: Drop-wise protocol	urea 4 M NaCl 300 mM HEPES 50 mM 2 L pH 7.5 At 4°C over night	(1) urea 2 M NaCl 150 mM HEPES 50 mM glycine 50 mM $MgCl_2$ 1 mM $CaCl_2$ 2.2 mM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % 2 L pH 7.2 At 4°C over night	(2) NaCl 150 mM HEPES 50 mM glycine 50 mM Sucrose 50 mM $MgCl_2$ 1 mM $CaCl_2$ 2.2 mM L-arginine 250 mM glutathione red. 5 mM glutathione ox. 0.25 mM Tween-20 0.005 % PEG3350 0.05% Triton X-100 0.05% 0.4 L pH 7.2 At 4°C over night	NaCl 150 mM Tris HCl 50 mM Tween-20 0.005 % pH 8.0 At 4°C for 4 h, Exchange to a fresh storage buffer, dialysis Over night

Table IV.2 Refolding protocol for the drop-wise method. It was used for the drop-wise method in order to refold KLK15 fl, KLK15 ntfl, and KLK15 S/A. This method was employed, because it is known to be a more effective way of refolding specific proteases.

IV.3.1.4 Incubation with enterokinase

Enterokinase is a serine protease involved in human digestion, belonging to the chymotrypsin-clan. It hydrolyzes a unique lysine-isoleucine peptide bond in trypsinogen as the zymogen enters the duodenum from the pancreas. Accordingly, the minimal amount of trypsin derived in this way activates more trypsinogen and the other zymogens (Mann et al., 1994; Light et al., 1989; Berg et al., 2002). Enterokinase is a specific protease that cleaves a protein after a lysine residue at its preferential recognition site, which is Asp-Asp-Asp-Asp-Lys (**fig.IV.13**) It will not cleave, if the recognition site is followed by proline. In order to activate the purified and refolded KLKs, they were treated with recombinant porcine enterokinase (Genscript, Piscataway, USA), resulting in the removal of the N-terminal histidine-tag plus the enterokinase site. The enzyme solution was incubated with 1 U enterokinase per 100 µg constructs at room temperature. Since the optimal incubation time was unknown, several digestion reactions were set up with different time points from 20 min to 20 h. In addition, temperature and enterokinase concentration were modified to optimize the digestion. Cleavage of the control protein by enterokinase was evaluated by SDS-PAGE. Accordingly, the enzyme/EK reaction was aliquoted and stored at -80°C.

Additionally, N-terminal sequencing of the cleaved KLKs was performed by the lab of Dr. J. Kellermann and Prof. Dr. F. Lottspeich at the Max-Planck-Institute for Biochemistry, Martinsried, Germany (section Protein Analysis).

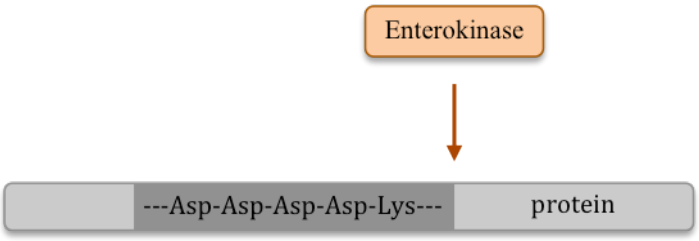


Figure IV.13 The preferred target site of enterokinase. The protease preferentially cleaves Asp-Asp-Asp-Asp-Lys. One U of enterokinase is defined as the amount of enzyme needed to cleave 50 µg of fusion protein in 16 h to 95% completion at 22°C in a buffer containing 25 mM Tris-HCl, pH 8.0.

IV.3.1.5 Enterokinase antibody

Enterokinase was removed employing an enterokinase removal kit (Sigma, USA). It is designed for the removal of bovine enterokinase from mixtures containing a fusion protein cleaved by the enzyme. The enterokinase removal kit consists of the antibody directed against enterokinase coupled to agarose. It was provided in a 50% (v/v) suspension containing 0.01 M phosphate buffered saline and 15 mM sodium azide. The washing buffer containing 0.4 M Tris-HCl, pH 7.4, 1 M NaCl and 0.04 M $CaCl_2$ was prepared and diluted 20 times. In order to capture 1 U of porcine enterokinase from the solution 50 µL of 50% suspension containing the antibody was utilized. For resuspension and washing the antibody-agarose was suspended in 50 µL 1x washing buffer per 10 µL of 50% anti-enterokinase suspension. As a following step the solution was centrifuged at 1020 rcf (1000 rpm) for 2 min at 4°C, and the supernatant was discarded. This step was repeated twice. Subsequently, the mixture containing the KLK, cleaved by the enzyme, was added and incubated for 1 h at room temperature on a gently shaking platform (Coulter Mixer). The reaction sample was put on ice for 5 min so as to improve the separation of the liquid phase and the agarose. Hereafter, the mixture was spun down (1000 rpm) for 2 min at room temperature. The cleaved and activated KLKs were recovered by separation of the liquid phase of the reaction from the anti-enterokinase agarose. Enterokinase was captured and remained on the bottom of the vial. SDS-PAGE was performed to check on the efficiency of the reaction.

IV.3.1.6 Benzamidine affinity column for trypsin-like KLKs

To further enhance purity and select active forms from a mixture of cleaved and uncleaved protein, active KLKs were recovered from the sample using an immobilized inhibitor of trypsin-like proteases. It is commercially available as benzamidine covalently bound to sepharose 6B (GE Health Care, Uppsala, Sweden), in a solution of 6% agarose in 20% ethanol. P-aminobenzamidine (PABA) is a synthetic inhibitor of trypsin and trypsin-like serine proteases and hence useful for separating active KLKs from their inactive form. P-aminobenzamidine was packed into a 100 mL column made of medical grade polypropylene, a biocompatible material that does not interact with biomolecules. To purify 1 mL of sample, 1 mL of 1 µg/µL benzamidine-sepharose was utilized. The column was washed and equilibrated at room temperature with a washing-binding buffer (10x column volume) containing 50 mM Tris HCl, pH 8, 300 mM NaCl and 0.005% Tween-20. Next, the sample was loaded on the column and the unbound proteases were washed with the same buffer. Accordingly, active KLKs that bound to the column were eluted competitively with an elution buffer containing 100 mM glycine, 500 mM NaCl, pH 3.5 and Tween-20 0.005% (5x column volume). After running an SDS-PAGE, the eluted protease was stored at -80°C. Subsequently, the column was washed and stored at 4°C.

IV.3.1.7 Protein blot from SDS-PAGE to PVDF-Membrane

In order to evaluate the cleavage products after enterokinase treatment, N-terminal sequencing was performed by the laboratory of J. Kellermann and Prof. Dr. F. Lottspeich at the Max-Planck-Institute for Biochemistry, Martinsried, Germany (section Protein Analysis). For this, proteins were transferred onto a PVDF membrane after electrophoresis separation with SDS-PAGE utilizing the semi dry electro-transfer procedure as described by Gershoni and Palade, 1983 (**fig.IV.14**). The gel containing the protein rested on the top of PVDF membrane. A sandwich of 6 pieces of paper filters was made that were cut in the size of the gel, three on the top and three on the bottom. The SDS gel was pre-wetted in 50 mM boric acid, pH 8.5 with 5% ethanol. The lower filters were soaked in buffer containing 20% ethanol and 50 mM boric acid, pH 8.5, and the upper filter in 5% ethanol and 50 mM boric acid. The PVDF membrane was briefly pre-wetted in 99% ethanol and then soaked in buffer, 20% ethanol and 50 mM boric acid. The sandwich was placed between two electrodes and air bubbles were removed. A voltage of 95 V, 2 mA per cm^2 gel and 10 W was

applied across the apparatus for 1.5 h and proteins thereby transferred onto the PVDF membrane. As a following step the PVDF membrane was incubated in 0.1% Coomassie Blue, 40% ethanol and 10% acetic acid for less than one min. Subsequently the membrane was destained in 40% ethanol and 10% acetic acid solution.

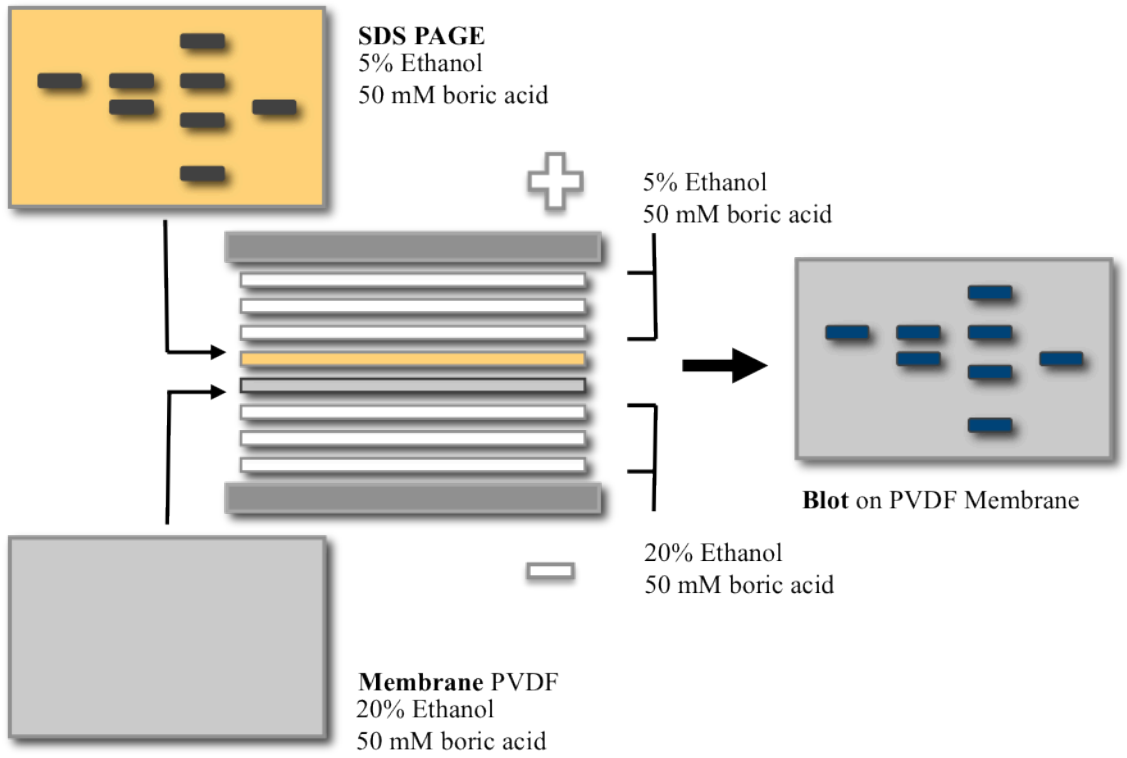


Figure IV.14 Electroblot to transfer proteins onto a membrane. This method uses a PVDF membrane after gel electrophoresis. It relies upon current and a transfer buffer solution to drive proteins onto a membrane. A stack is put together in the following order from anode to cathode: three sheets of filter paper soaked in 20% ethanol and 50 mM boric acid, PVDF membrane, gel and three sheets of filter paper soaked in 5% ethanol and 50 mM boric acid. It is essential that the membrane is located between the gel and the anode, as the current and sample will be moving in that direction. Once the stack is prepared, it is placed in the transfer system, and a current of 95 V is applied for 1.5 h. The electrode plate of the apparatus is made either of platinum coated stainless steel or carbon and graphite.

IV.3.1.8 Enzymatic kinetic studies

In order to evaluate enzymatic activity of purified, refolded and activated KLKs, fluorogenic 7-amino-4-methyl coumarin (-AMC) substrates with a specific amino acid sequence were utilized (**table IV.3**). The synthetic substrates were purchased from Bachem, Weil am Rhein, Germany. Kinetic parameters of the KLK4, 8 and 15 were assessed in storage buffer: NaCl 100 mM, Tris HCl 50 mM, Tween-20 0.005 %, pH 8.0 at room temperature in a final volume of 250 µL. The active enzyme with a concentration of 1-50 nM was added to

the buffer and briefly incubated at room temperature. Subsequently, the fluorescent substrate (1 µL of a 5 mM solution) was added. Fluorescence resulting from cleavage of AMC-substrates was measured with multilabel counter (355 nm, emission: 460 nm, VICTOR³ 1420 by Perkin Elmer, Waltham, Massachusetts, USA).

Substrate name	Amino acid sequence	Recommended serine protease
I-1120	Boc-Val-Pro-Arg-AMC	Trypsin-like
I-1400	Boc-Phe-Ser-Arg-AMC	Trypsin-like
I-1595	Boc-Gln-Gly-Arg-AMC HCl	Trypsin-like
I-1395	Suc-Leu-Leu-Val-Tyr-AMC	Chymotrypsin-like

Table IV.3 Amino acid sequence of fluorogenic 7-amino-4-methyl coumarin (-AMC) substrates. These were used to study the activity of KLKs. Boc-, Di-*tert*-butyl dicarbonate-; Suc-, Succinyl-.

IV.3.1.9 Effects of KLK4, 8 and 15 on pro-uPA

In order to evaluate interplay of KLKs with pro-uPA, KLK4, 8 and 15 were incubated with the protease precursor. The assay was performed for different time periods, in a storage buffer, containing 100 mM NaCl, 50 mM Tris HCl and 0.005 % Tween-20, pH 8.0. The ratio of pro-uPA to KLK was 100:1. For comparison, the reaction was incubated at 37°C and room temperature. As a negative control, pro-uPA alone was treated under the same conditions. The results were assessed by SDS-PAGE. The resulting samples were then stored at -20°C for further analysis.

IV.3.1.10 Activity studies using fluorogenic substrates: Cleavage of PAR peptides by KLKs

In order to evaluate cleavage of PAR peptides by KLKs, short peptides (7 amino acids) containing the receptor's activation site of PAR1, 2 and 4 were utilized (**table IV.4**). Those short peptides are labeled with both a fluorochrome and a quencher. Normally the emerging fluorescent energy is absorbed by the quencher group. Cleavage of the peptide by proteases separates the quencher from fluorochrome and thus enables fluorescence to be detected. A wavelength of 355 nm triggers the emission of a photon at 460 nm (Didenko et al., 2006). Generally, fluorogenic substrates were stored protected against light.

PAR	Amino acid sequence
1	Fl- Leu - Asp - Pro - Arg - Ser - Phe - Leu - Leu -Q
2	Fl- Ser - Lys - Gly - Arg - Ser - Leu - Ile - Gly -Q
3	Fl- Pro - Ala - Pro - Arg - Gly - Tyr - Pro - Gly -Q

Table IV.4 Amino acid sequence of short PAR peptides with the receptor's activation site. They were used to study the activity of KLKs. Fl, fluorescein; Q, quencher.

The assay was performed in a final volume of 200 μL at room temperature, using a buffer that contains 135 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM Glucose and 11 mM HEPES, pH 7. Subsequently, the protease (either thrombin, trypsin, KLK4 or KLK8), as well as the substrate (PAR1-, PAR2-, or PAR4-short peptide, concentration of 6.5 μM) were added to the sample. For fluorogenic measurements, samples were mixed on a black Optiplate 96F (Perkin Elmer, Waltham, Massachusetts, USA) and solutions were added in the following order: buffer, protease sample and, finally, the substrate. Fluorescence signals were measured on a fluorometer (VICTOR³ 1420 multilabel reader by Perkin Elmer, USA) immediately using an excitation of 355 nm and emission of 460 nm.

V Experiments and results

V.1 Cloning, expression and purification of KLK4, 8 and 15

Naturally, KLKs are expressed as single-chain pre-pro-enzymes, from which the pre-signal sequence (16 to 30 amino acids) is removed during secretion. The short pro-domain of the zymogen is cleaved off by proteases resulting in an active enzyme. The mature KLK contains a functionally active catalytic triad comprised of histidine (H), aspartic acid (D) and serine (S) (Emami et al., 2007). In order to purify recombinant KLKs, fusion genes were produced, that encode a synthetic pro-form of the KLK protease with an N-terminally located his-tag followed by an enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys↓). The construct was inserted into the bacterial expression-vector pQE-30 and the recombinant protein expressed by M15 *E. coli* cells. Inclusion bodies were solubilized utilizing guanidinium-HCl. The raw lysate containing the recombinant protein, was then purified by Ni^{2+}-NTA affinity chromatography using the his-tag to capture the recombinant KLKs.

V.2 Purification of KLK proteases by Ni^{2+}- NTA affinity

To isolate the recombinant proteins, lysed cells that expressed KLK4, 8, 15 fl or 15 ntfl were fractionated by affinity purification using the his-tag of the recombinant protein. The raw lysate was incubated with Ni^{2+}-NTA-agarose, washed extensively using 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris HCl, pH 8, with decreasing pH (8.0 – 6.0) in order to remove non-specifically bound bacterial proteins. Finally, the protein of interest was eluted from the resin at a further reduced pH (5.4 - 4.0). At lower pH, the histidine residues become protonated and thus the his-tagged protein dissociates from the Ni^{2+}-NTA-agarose. The purity and amount of protein was assessed by SDS-PAGE **(fig.V.1)**. Best yield of the protein of interest was found at pH 4.5. The resulting KLK solutions were stored at 4°C for further analysis and refolding.

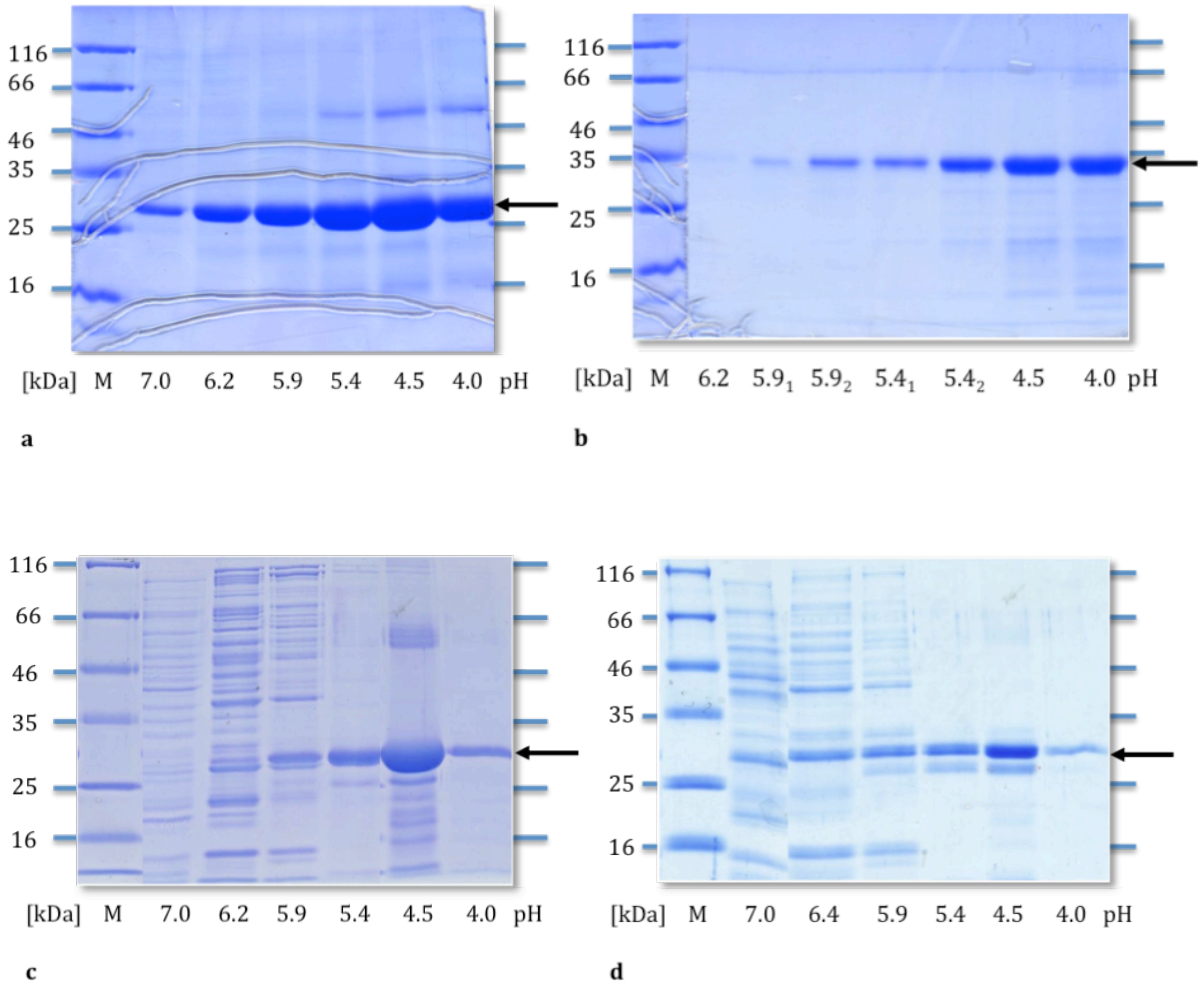


Figure V.1 Affinity purification of recombinant KLK proteases by Ni^{2+}-NTA agarose. A: KLK4, b: KLK8, c: KLK15fl and d: KLK15 ntfl. The arrows indicate a protein with a molecular weight between 35 and 25 kDa, most certainly the protein of interest. The samples from washing steps (pH 7.0 – 5.9) contain various proteins from *E. coli*, which were removed from the column, while most of the his-tagged proteins were still binding. By decreasing the pH, recombinant KLKs dissociated from the NTA-agarose. Highest concentration of the protein of interest can be found at pH 4.5. Proteins were separated by 12% SDS-PAGE and subsequently Coomassie-stained.

V.3 Refolding

As the proteins were expressed in bacterial cells, resulting inclusion bodies had to be solubilized by guanidinium-HCl and kept in their denaturated form by urea. Disulfide bonds were formed in a rather slow process by utilizing a redox pair of reduced glutathione (GSH) and oxidized glutathione (GSSG). Refolding procedures were performed in a dialysis bag with Spectra/Por membranes (MWCO: 12 -14,000 kDa) at 4°C on a magnetic stirrer at pH 8.0. The denatured protein with a concentration between 0.1 and 0.05 µg/µL was diluted in a large volume of refolding solution to prevent aggregation and precipitation. Afterwards

centrifugal concentration devices (Amicon Ultra-15 centrifugal filter unit by Millipore, France) were used to concentrate the refolded KLK. The amount of soluble recombinant KLK was assessed by SDS-PAGE **(fig.V.2)**.

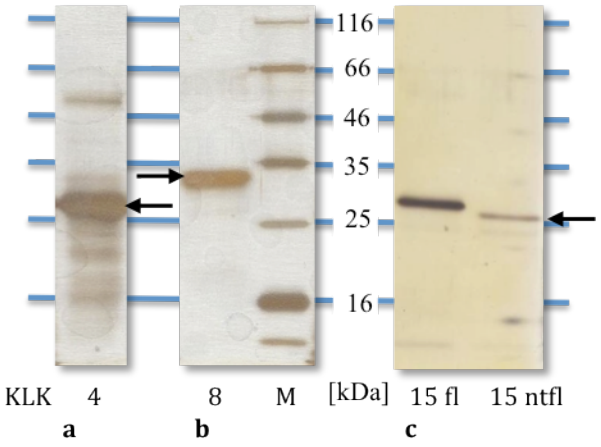


Figure V.2 Refolded KLK4, 8, 15 fl and 15 ntfl. Arrows are indicating the protease of interest, which displays a molecular weight between 25 and 35 kDa. Samples display a purity >95%. Proteins were separated by 12% SDS-PAGE and subsequently silver-stained.

Refolded KLK4 and 8 exhibited activity, as measured with fluorogenic substrates after activation with enterokinase. In contrast, KLK15 fl and ntfl were refolded unsuccessfully using various protocols. An increasing amount of precipitated protein was noted and the proteases did not exhibit any activity during enzymatic kinetic studies. An attempt to refold KLK15 fl and ntfl employing the more elaborate drop-wise method was made, as this is known as an effective way to refold certain proteases. However, in this case no active KLK15 fl or ntfl could be produced. Though it was assumed that correctly folded KLKs are soluble and therefore do not precipitate, in reality it is possible that precipitation related to concentration of the protein, temperature and buffer composition occurred. In the case of KLK15 fl, ntlf and S/A high amounts of the construct precipitated after refolding, resulting in significant losses of material. For example, 5 mL of purified KLK15 fl with a concentration of 0.13 µg/µL was refolded resulting into 1 mL of a 0.033 µg/µL solution. In summary, the preparation of soluble, refolded KLK4 and KLK8 was successful, while production of refolded KLK15 fl and KLK15 ntfl in larger amounts was unsuccessful.

V.4 Activation of recombinant KLK proteases by enterokinase

100 µg purified and refolded KLKs were treated with 1 U recombinant porcine enterokinase (Genscript, Piscataway, USA) that cleaves proteins on the C-terminal side of a lysine residue at its preferred recognition site, which is Asp-Asp-Asp-Asp-Lys. This results in the removal of the N-terminal his-tag and the enterokinase site, leaving the mature KLK without any additional amino acid residues (**fig.V.3**).

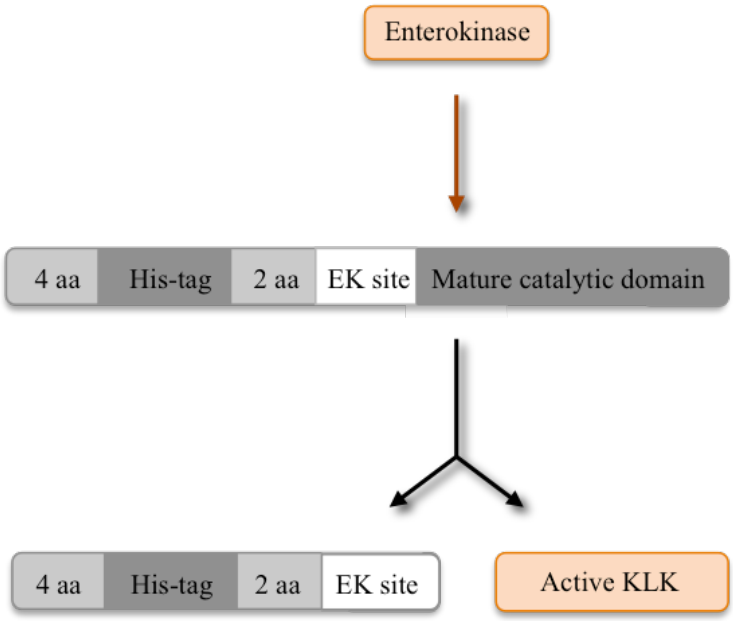


Figure V.3 Removal of the his-tag by enterokinase. EK cleaves the KLK construct specifically after lysine residues. Its recognition site is Asp-Asp-Asp-Asp-Lys. Cleavage results in active KLK lacking the his-tag and the EK-site.

Prior to incubation of enterokinase with the construct, activity of the proteinase was verified using a cleavage substrate specifically for enterokinase (Genscript, Piscataway, USA). A sample of Genscript cleavage substrate (1 µg/µL) was incubated with 1 U enterokinase per 10 µg of the protein mixture for 16 h at 37°C. Samples were run on SDS-PAGE, which showed that recombinant porcine enterokinase cleaves the control and is therefore active (**fig.V.4**). In case of activation of KLK proteases, various cleavage reactions were set up with different time points from 20 min to 20 h, while temperature and enterokinase concentration were modified to optimize the digestion. Cleavage of the recombinant proteins was evaluated by SDS-PAGE. Accordingly, the enzyme/EK reaction was aliquoted and stored at -80°C. N-terminal sequencing, performed with the cleaved KLKs by the laboratory of

Dr. J. Kellermann and Prof. Dr. F. Lottspeich at the Max-Planck-Institute for Biochemistry, Martinsried, Germany (section Protein Analysis), verified that incubation of KLK4, 8, 15 fl and 15 ntfl resulted in the formation of the mature N-terminus of each KLK.

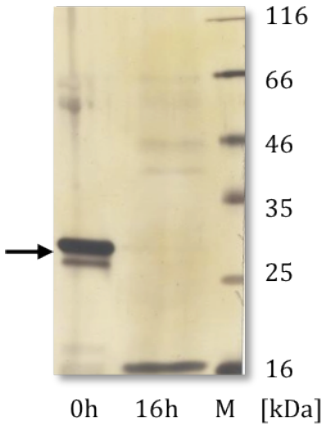


Figure V.4 Verification of the activity of enterokinase using a cleavage control. Arrow indicates 1 µg of cleavage control (Genscript, Piscataway, USA) with a molecular weight of around 30 kDa, which was then incubated with 0.1 U enterokinase for 16 h. Cleavage results in bands with a molecular weight less than 16 kDa.

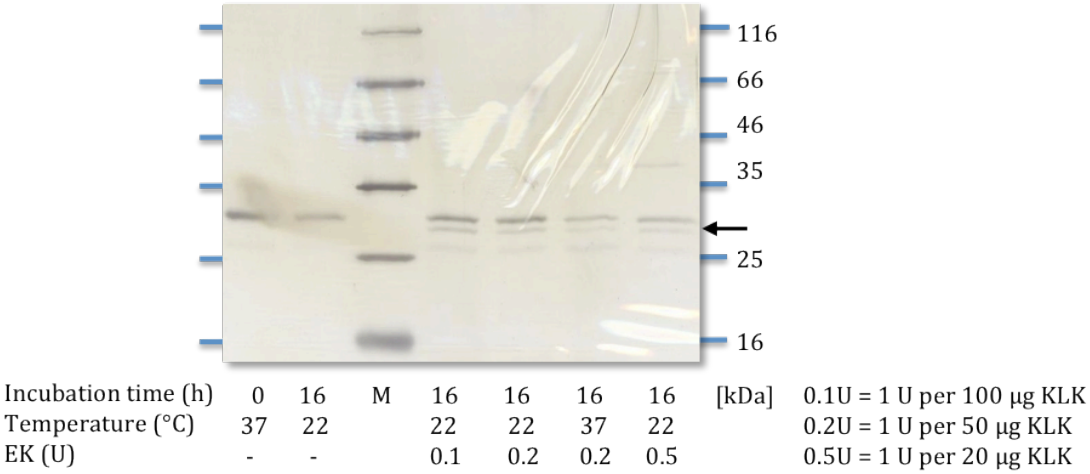


Figure V.5 Digestion of KLK15 fl with porcine enterokinase and time course optimization. The samples were incubated at room temperature or at 37°C. Different amounts of enterokinase were used to assess and compare its efficacy in cleaving KLKs. Arrow indicates cleaved forms, which lack the his tag and the EK cleavage site. Cleaved proteins display a slightly lower molecular weight.

Figure V.5 shows that incubation of KLK15 fl alone for 16 h at 22°C does not lead to new bands besides that of KLK15 fl. This indicates that (at 22°C) KLK15 fl is not able to auto-activate as this would show a smaller peptide after incubation. It is worth noting that

increasing concentration of enterokinase enhances the digestion of the construct, as its signal decreases when higher concentrations of enterokinase are used. Interestingly, incubation of KLK15 fl at 37°C seems to lead to a weaker signal compared to 22°C, possibly as a result of degradation of the protein at the higher temperature. Further incubation assays with enterokinase were thus performed at 22°C and not at 37°C. For activation of the constructs 1 U enterokinase per 100 µg KLK was utilized. In a similar approach to activate KLK15 ntfl (**fig.V.6**), it has been shown that no cleaved form of the protein occurs after incubation on its own for 16 h. The results matched those of KLK15 fl, incubation of KLK15 ntfl at 37°C leading to a weaker signal compared with that at 22°C, and increasing concentration of enterokinase enhances the digestion of KLK15 ntfl.

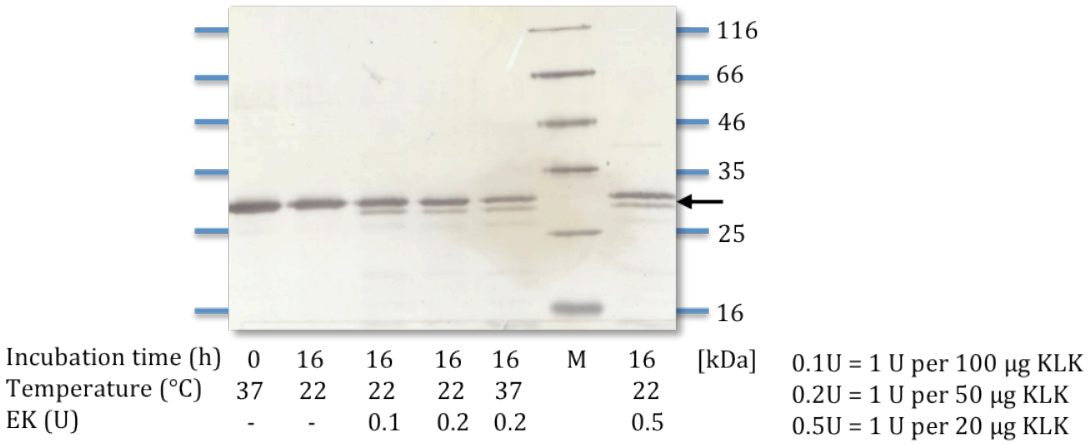


Figure V.6 Digestion of KLK15 ntfl with porcine enterokinase and time course optimization. The samples were incubated at room temperature or at 37°C. Different amounts of enterokinase were used to assess and compare its efficacy in cleaving KLKs. Arrow indicates cleaved forms which lack the his tag and the EK cleavage site. Cleaved proteins display a slightly lower molecular weight

V.5 Site-directed mutagenesis: KLK15 S/A

Incubation of KLK15 fl and ntfl with EK leads to decreasing concentrations of both the recombinant proteins. Two possibilities could explain this: first, EK does not only cleave the pro-peptide, but also cleaves at other sites of the KLK15 molecule; second, the recombinant KLK15 proteases are active and undergo autolysis. In order to rule out the latter, *in vitro* mutagenesis of KLK15 was performed, exchanging the active site serine for alanine to inactivate the protease.

V.5.1 Cloning: KLK15 S/A

In order to generate site-directed mutagenesis of KLK15, the pQE-30 derived KLK15 expression plasmid was used as a template and PCR with mutated primers was performed. Subsequently, plasmids were transformed into *E. coli*. DNA was isolated and restriction analysis on the mutated plasmids. The DNA was then loaded on a 1% agarose gel (**fig.V.7**). The DNA isolated from clones 2 to 4 appeared as two bands on the gel of the expected MW, implying that the side-directed mutagenesis was successful and the plasmid contained the desired mutation. *In vitro* mutagenesis was indeed verified by sequencing performed on DNA extracted from clone 3 (Metabion, Germany). The non-mutated plasmid of clone 1 was visualized by a single band, indicating that the plasmid did not contain the mutation.

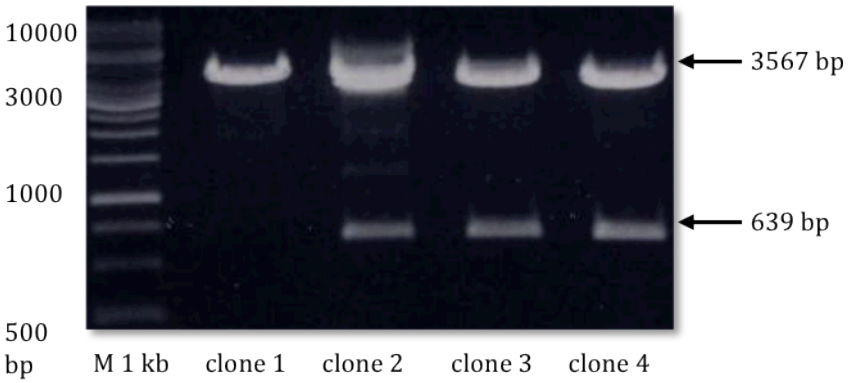


Figure V.7 Restriction analysis of candidate clones. Four plasmids were run on a 1% agarose gel at 90 V after digestion with Nae I. Clones 2 to 4 have the mutation that contains a novel restriction site for Nae I and therefore generate two fragments. Digestion of DNA derived from clone1 resulted in only one fragment, which indicates that the plasmid does not contain the mutated DNA. The DNA size marker is a 1 kb ladder (ladder 25_2030, peqlab Biotechnology GmbH, Germany). The gel was stained with ethidium bromide.

V.5.2 Expression of KLK15 S/A in M15 *E. coli* cells

The mutated plasmid was transformed into M 15 *E. coli* cells, expression induced by IPTG and proteins released from the cells using cracking buffer (**fig.V.8**). Finally, clone 3 was chosen for further production and purification of the KLK15 S/A, as it had the highest levels of expression.

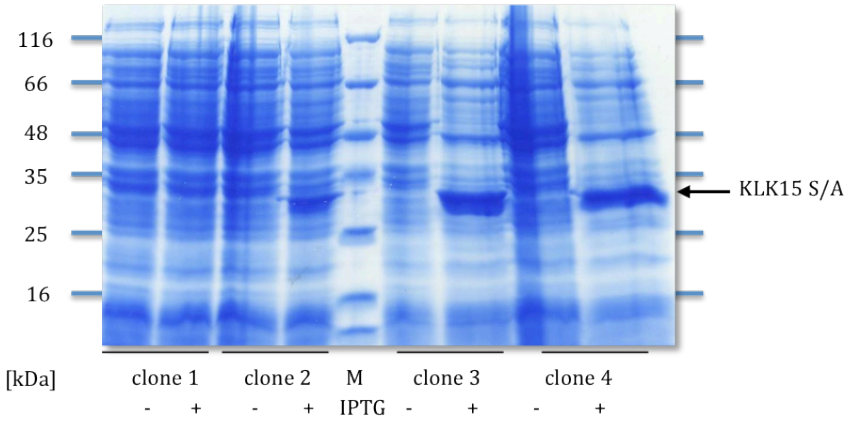


Figure V.8 Expression analysis of KLK15 S/A in M15 cells. Overexpression of a protein with a molecular weight between 35 and 25 kDa is observed, which is most certainly KLK15 S/A. Interestingly, clone 1 does not appear to express KLK15 S/A after induction. Proteins were separated by 12% SDS-PAGE and subsequently Coomassie-stained.

V.5.3 Purification and refolding of KLK15 S/A

The recombinant KLK protease was purified by Ni^{2+}-NTA agarose and refolded accordingly **(fig.V.9)**.

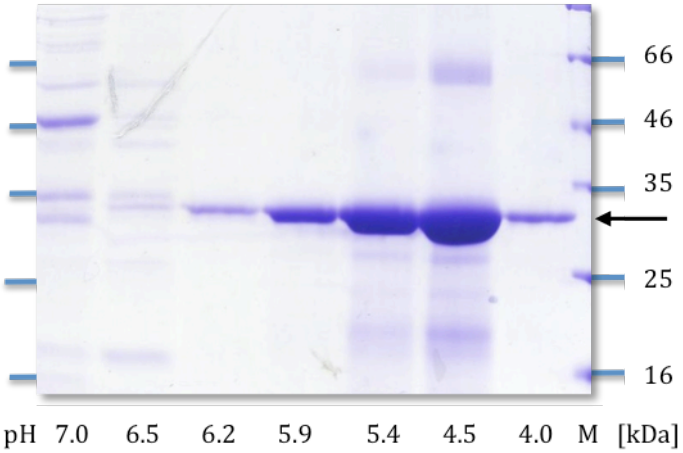


Figure V.9 Purification of recombinant KLK15 S/A with Ni^{2+}-NTA agarose. The arrow indicates a protein with a molecular weight at about 30 kDa, most certainly the protein of interest. The samples from washing steps (pH 7.0 – 5.9) contain various proteins from *E. coli*, which were removed from the column while his-tagged proteins were still binding. By decreasing the pH, recombinant KLKs dissociated from the NTA-agarose. Highest concentration of the protein of interest can be found at pH 4.5. Proteins were separated by 12% SDS-PAGE and subsequently Coomassie-stained.

V.5.4 Incubation of KLK15 S/A using enterokinase

Refolded KLK15 S/A was subsequently incubated with enterokinase. In line with previous observations of similar incubations, the concentration of the protease decreases over the time-course **(fig.V.10)**.

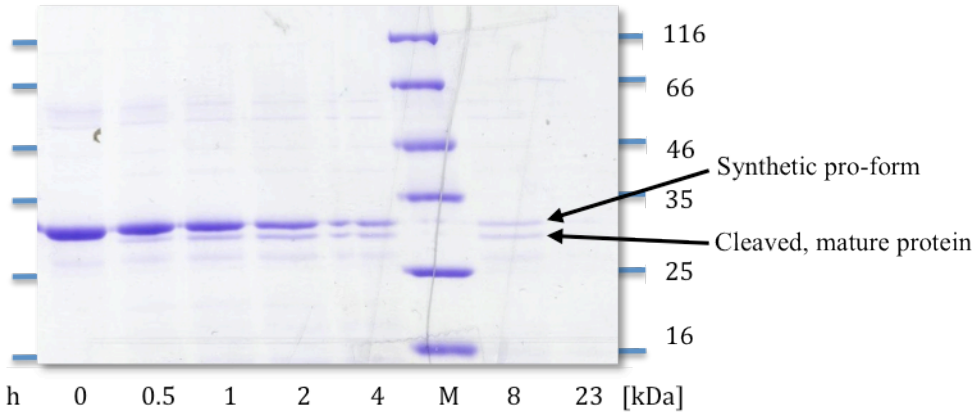


Figure V.10 Cleavage of recombinant KLK15 S/A by enterokinase. 0.2 U enterokinase per 10 µg construct was incubated at 37°C. apparently the optimal incubation period is 4 h. Proteins were separated by 12% SDS-PAGE and subsequently Coomassie-stained.

V.5.5 Summary of enterokinase incubation

A panel of enterokinase digestions encompassing a range of U and incubation times was used to find the optimal conditions to produce a mature KLK (**table V.1**).

Conditions	Incubation time (h)	Temperature (°C)	Enterokinase (U)
KLK4	18	22	0.1
KLK8	18	22	0.1
KLK15 fl	2	22	0.1
KLK15 ntfl	4	22	0.1
KLK15 S/A	4	22	0.1

Table V.1 Optimal conditions for cleaving off the synthetic pro-peptides of each KLK with porcine enterokinase.

V.6 Removal of enterokinase using antibodies

To further purify the samples containing potentially activated KLKs, the tiny amounts of enterokinase were removed employing an enterokinase removal kit (Sigma, USA). The kit

provides an immobilized antibody directed against enterokinase. It is designed for the removal of bovine enterokinase from mixtures containing a fusion protein cleaved by the enzyme. In order to capture 1 U of porcine enterokinase from the solution, 50 µL of 50% slurry containing the antibody was utilized.

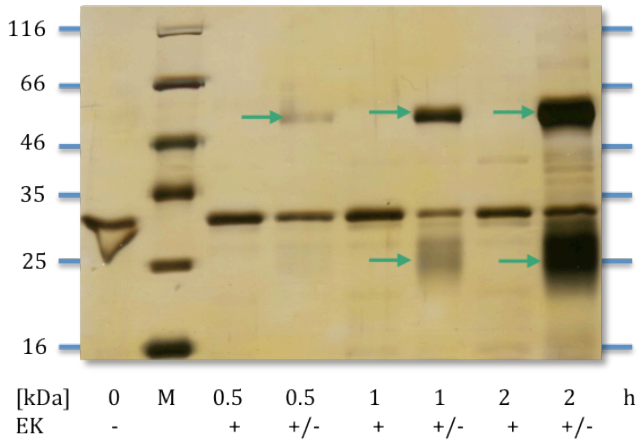


Figure V.11 Digestion of refolded KLK15 fl with enterokinase. In some samples enterokinase was removed utilizing the enterokinase removal kit (Sigma, USA), indicated by (+/-). Green arrow indicates contamination from anti-enterokinase agarose. Those samples that were treated with anti-enterokinase agarose display an additional band with a molecular weight between 66 and 46 kDa, most likely a contaminant from the enterokinase removal kit. Despite elaborate washing and centrifugation steps, samples treated with the antibody contained this contaminant. Proteins were separated by 12% SDS-PAGE and subsequently silver-stained.

Figure V.11 compares samples in which enterokinase was removed with other samples still containing the proteinase. It is worth noting that those samples that were treated with anti-enterokinase agarose display an additional band with a molecular weight between 66 and 46 kDa, probably a contamination from enterokinase removal kit. Despite elaborate washing and centrifugation steps, samples treated with the antibody contained this contaminant.

V.7 Affinity column purification with benzamidine

Purified, active KLKs were recovered from the sample using commercially available benzamidine sepharose 6B (GE Health Care, Uppsala, Sweden). P-aminobenzamidine is a reversible inhibitor of trypsin-like enzymes, first described in 1973 and used for purification of thrombin and trypsin (Beynon et al., 2001). Active KLKs were bound to the column at pH 8 and eluted competitively with an elution buffer at pH 3.5. Different fractions of the benzamidine elution were run on SDS-PAGE and the eluted protease stored at -80°C.

To rule out the possibility that active KLKs did not bind to benzamidine and got washed away, each fraction was tested for activity towards the fluorogenic substrates. **Figure V.12** alludes that after loading the benzamidine column with a sample, a large amount of protein does not bind to the resin and gets washed away, as shown in washing steps W1 to W10. In addition, the loading flow through of the KLK8 sample, presents a strong band on the gel. Interestingly, the elution fractions E3 and E4 show a strong signal of cleaved KLK8, indicating that at this step active KLK8 was eluted from the column. This could mean that the column capacity was insufficient or that it was overloaded with protein. Affinity column purification using benzamidine was performed utilizing the same materials and protocol as those for activated KLK4.

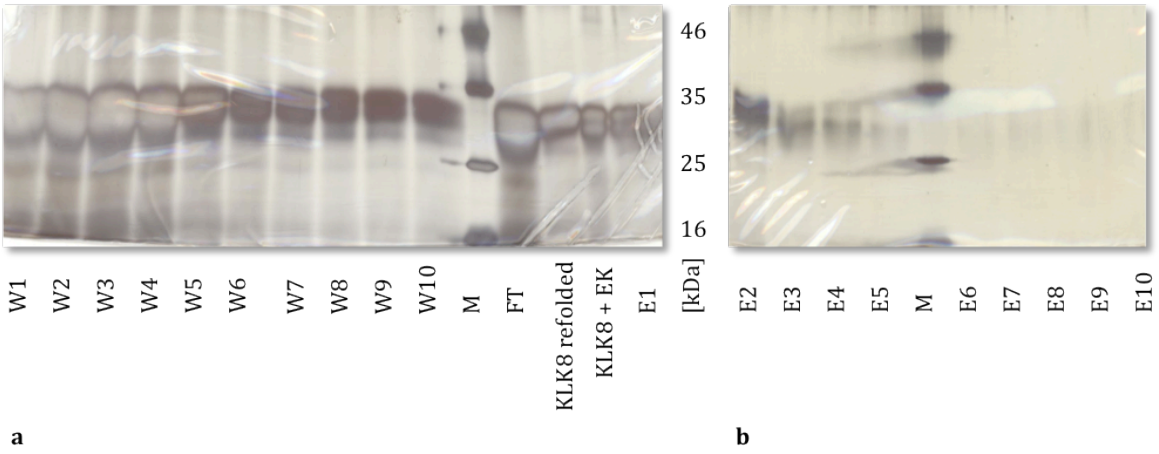
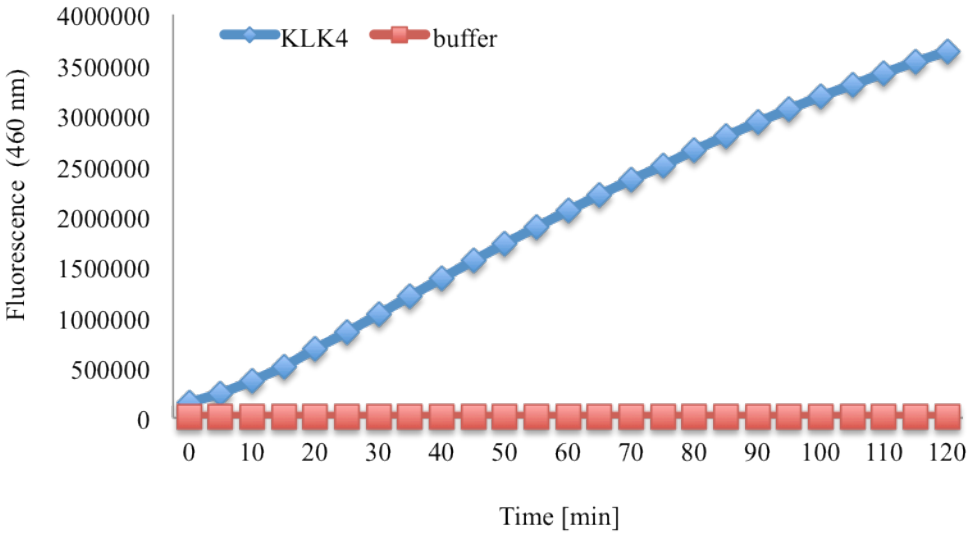


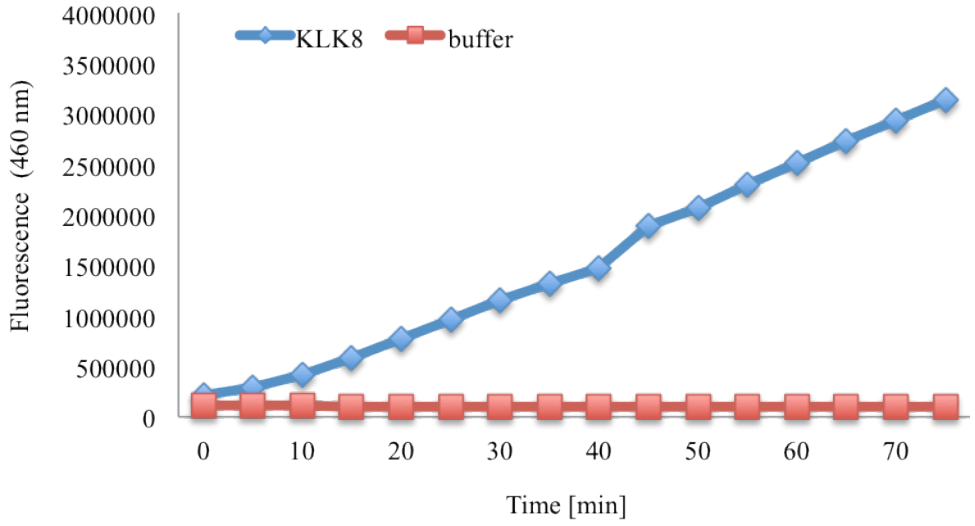
Figure V.12 Purification of active KLK8 using benzamidine sepharose. W = washing fraction, FT = flow through after loading the benzamidine column, E = elution fraction. Washing fractions seem to contain a large amount of KLK8, both cleaved and uncleaved, that did not bind to the column. The elution fractions display decreasing concentrations of KLK8. It is worth noting that elution fractions E3 and E4 seem to contain more cleaved KLK8, indicating that at this step some active KLK8 was eluted from the column. Proteins were separated by 12% SDS-PAGE and subsequently silver-stained.

V.8 Enzymatic kinetic studies

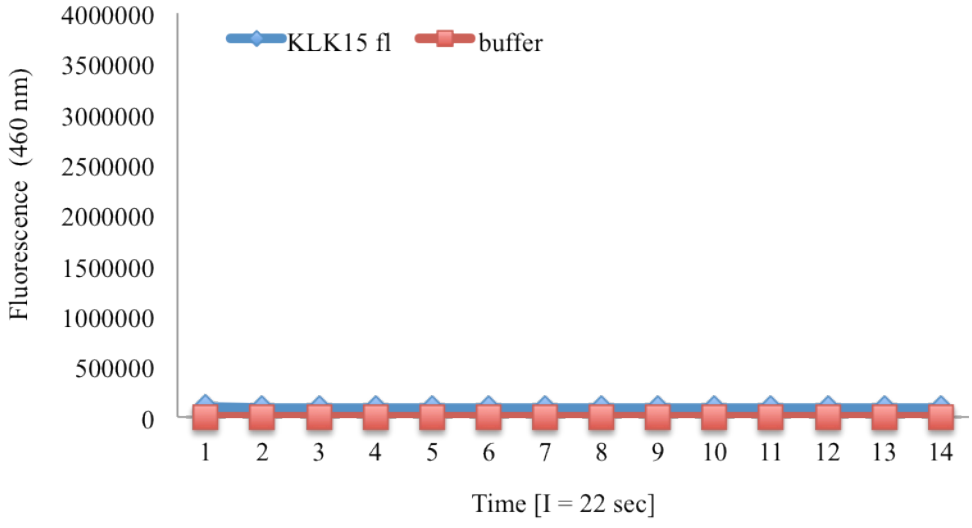
Enzymatic activity of purified, refolded and activated KLKs, was tested towards fluorogenic 7-amino-4-methyl coumarin (-AMC) substrates. Active KLK4 and 8 (**fig. V.13 and V.14**) showed an increasing fluorescence signal using substrates specific for either trypsin-like serine proteases (I-1120, I-1400 and I-1595). In contrast KLK15 fl and ntfl (**fig. V.15, 16**) did not show any activity towards fluorescent substrates. KLK15 S/A (**fig. V.17**) was expected not to display any activity.


KLK4
buffer
Fluorescence (460 nm)
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0 10 20 30 40 50 60 70 80 90 100 110 120
Time [min]

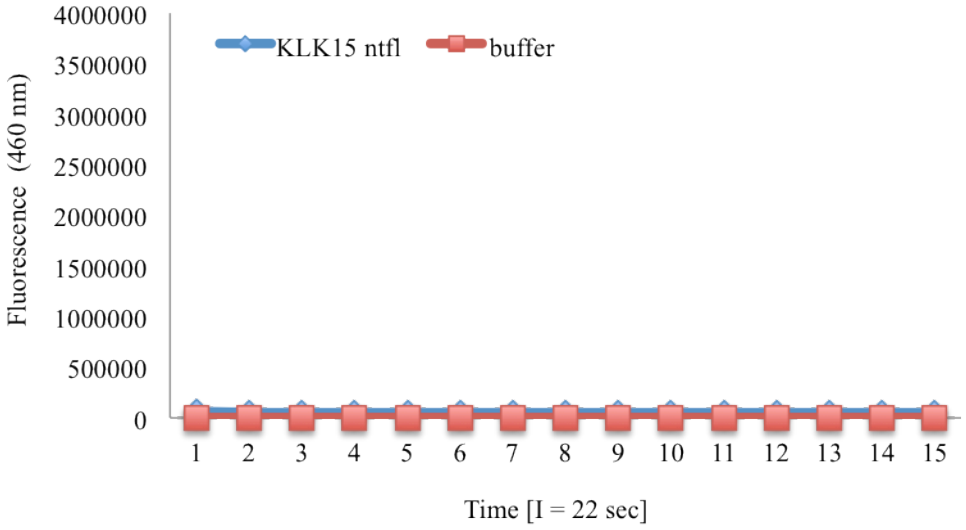
V.13


KLK8
buffer
Fluorescence (460 nm)
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0 10 20 30 40 50 60 70
Time [min]

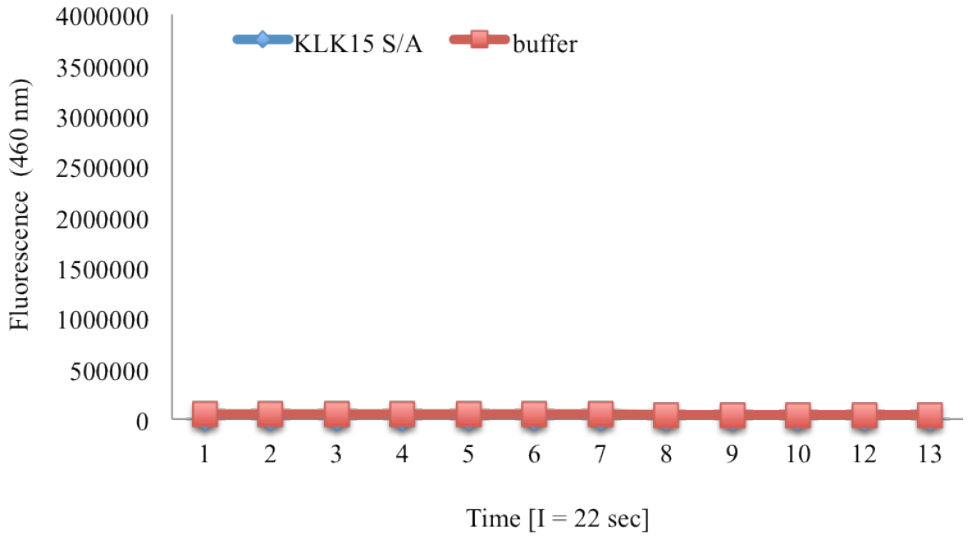
V.14


KLK15 fl
buffer
Fluorescence (460 nm)
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time [I = 22 sec]

V.15



V.16



V.17

Figure V.13 – 17 KLKs incubated with fluorescent substrates. Fig.V.13 shows KLK4 towards I-1120 which is Boc-Val-Pro-Arg-AMC, V.14 KLK8 towards I-1120; V.15 KLK15 fl towards I-1595 which is Boc-Gln-Gly-Arg-AMC HCl. In Fig.V.16 KLK15 ntfl towards I-1595 and V.17 KLK15 S/A towards I-1120. Incubation at excitation 355 nm and emission 460 nm.

V.9 Cleavage of PAR peptides by KLKs

As some KLKs, such as KLK2 and 6 (Ramsay et al., 2008; Hollenberg et al., 2008; Mize et al., 2008) have previously been found to cleave and activate proteinase-activated receptors (PARs), short peptides (7 amino acids) mimicking the receptor activation sites of PAR1, 2 and 4 were incubated with KLK4 and KLK8 in order to investigate whether these two KLK proteases may also represent candidates for activators of PARs.

V.9.1 Proteases such as thrombin and trypsin cleave PARs

Proteinase-activated receptors (PARs) are seven pass membrane-spanning, G-protein-coupled receptors, known to be involved in proliferative signaling of cancer cells (**fig.V.18**). Their activation, cleavage of an N-terminal extracellular sequence, reveals a tethered ligand, which subsequently binds to a receptor domain triggering activation of PAR. Cleavage downstream of the tethered ligand sequence may disarm the receptor, preventing its activation by other enzymes. PARs activation results in cytosolic calcium mobilization and intracellular signaling, followed by a rapid and transient desensitization of the receptor via endocytosis (Ramsay et al., 2008; Mize et al., 2008). Thrombin and trypsin are known to cleave PARs eliciting activation of the receptors (Darmoul et al., 2003; Vu et al., 1991).

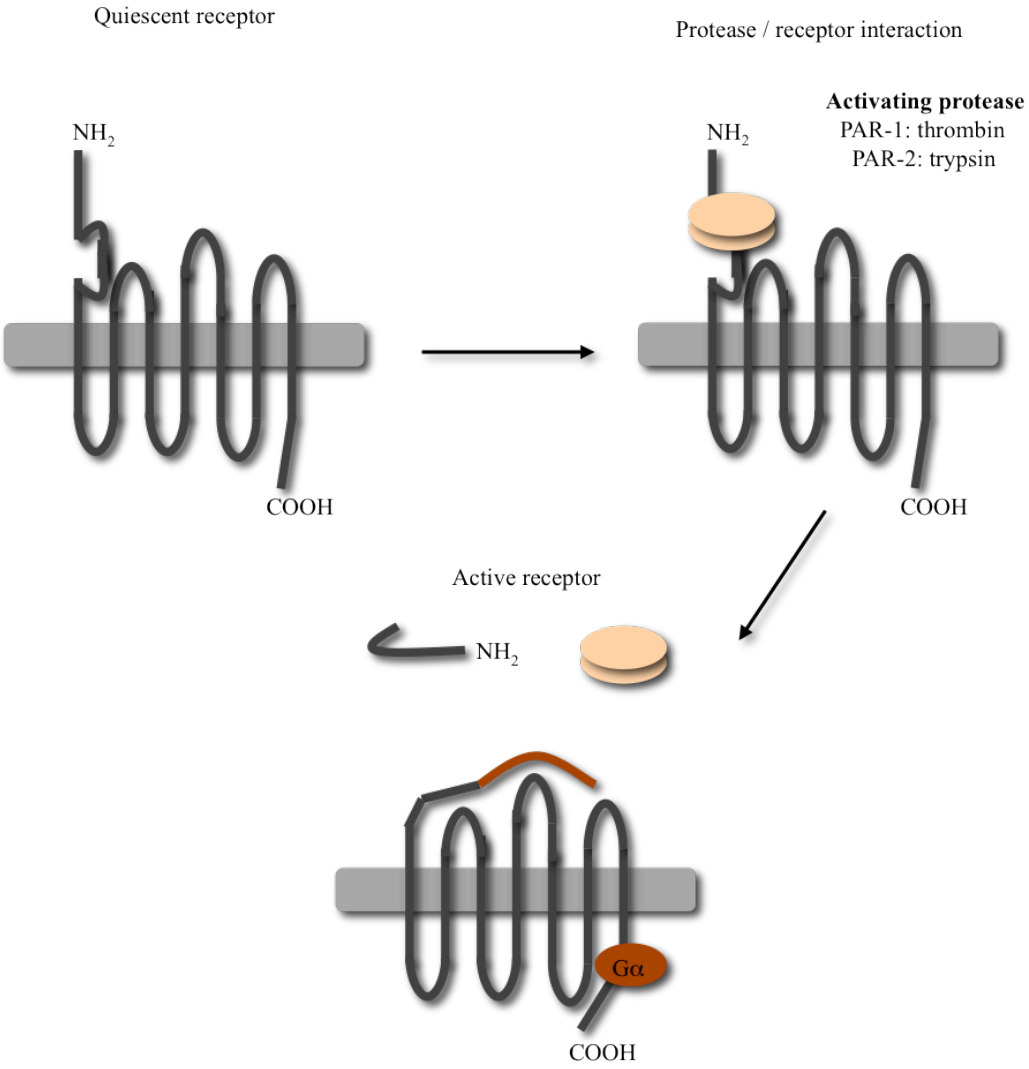
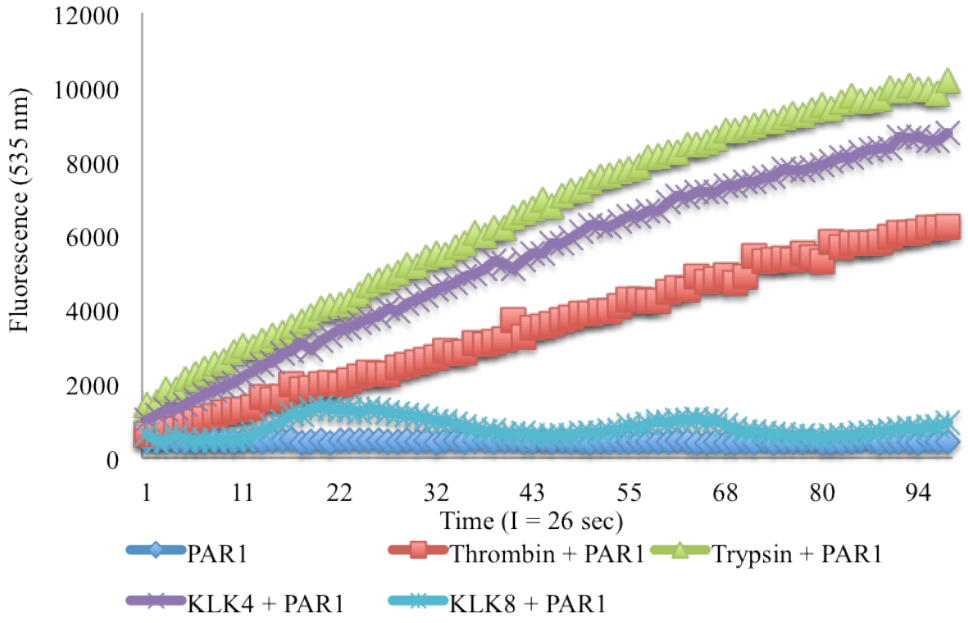


Figure V.18 PARs activation is mediated through proteases. Upon processing at their activation cleavage site by proteases (e.g. thrombin for PAR-1 and trypsin for PAR-2) the new aminoterminal region binds to the PAR's second extracellular loop, which then activates the receptor.

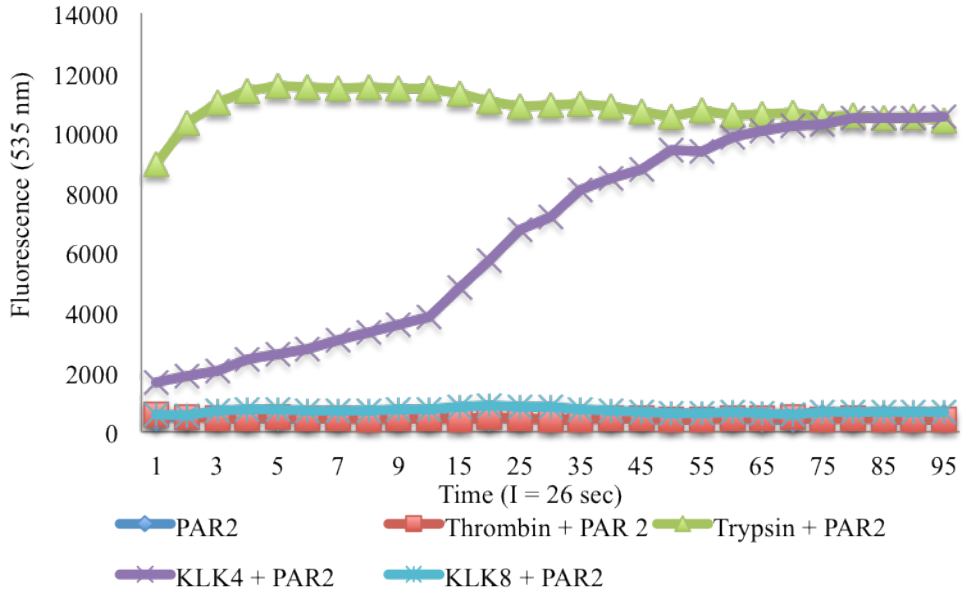
V.9.2 Cleavage of KLKs upon PARs: Activity studies using fluorogenic substrates

Cleavage of PAR1, 2 and 4-derived short peptides (7 amino acids) by proteases such as KLK4 and KLK8 was evaluated. The peptides contained an N-terminally located quencher and a C-terminally located fluorochrome group. If the peptide is cleaved, the fluorochrome is released, as well as the quencher and thus fluorescence can be detected. Short peptides and the protease (either thrombin, trypsin, KLK4 or KLK8) were mixed on a black Optiplate 96F (Perkin Elmer, Waltham, Massachusetts, USA). The fluorescence signal was measured on a fluorometer (VICTOR³ 1420 multilabel reader by Perkin Elmer, USA) using an excitation of

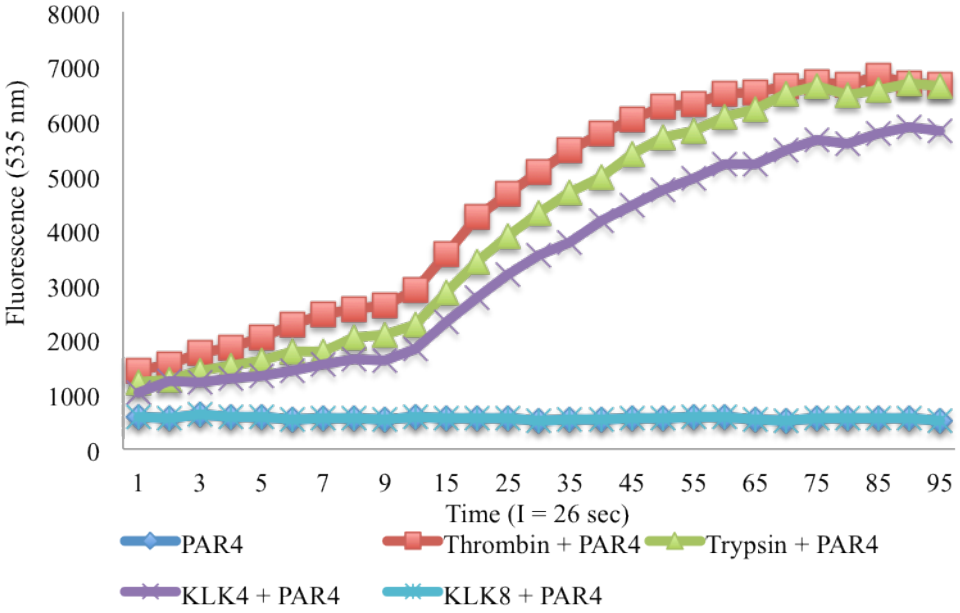
355 nm and emission of 460 nm. Results showed (**fig.19 a-c**) that the PAR1- and PAR4-peptides are cleaved efficiently by thrombin, trypsin and KLK4, as an increasing fluorescence signal was measured. Interestingly, the PAR2-peptide seems to be activated only by trypsin and KLK4, but not by KLK8 or thrombin, whereas PAR4-peptide was cleaved by trypsin, thrombin and KLK4, but not by KLK8.



a



b



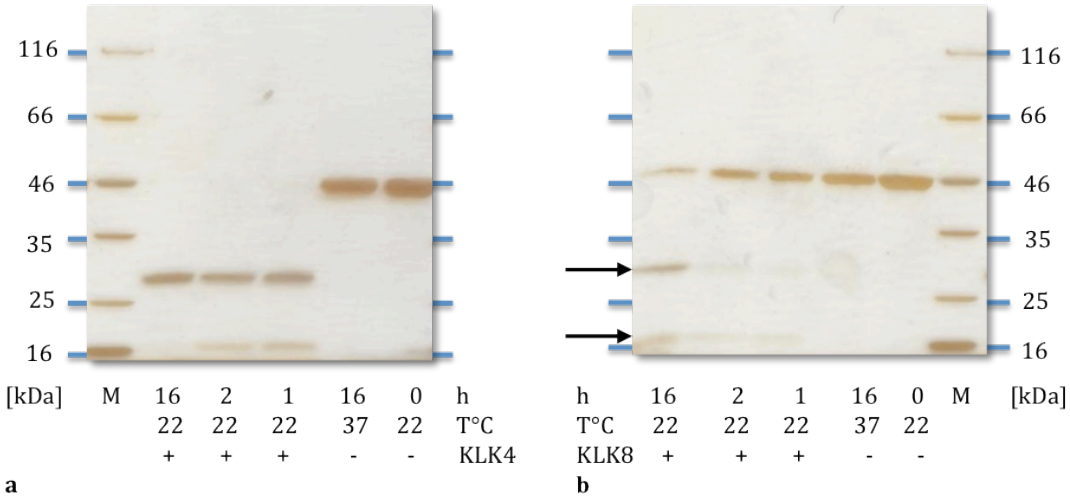
c

Figure V.19 PAR-peptides cleaved by proteases. Fig.a: PAR1-peptide, b: PAR2-peptide and c: PAR4-peptide incubated with thrombin (1.0 U/mL), trypsin (0.025 U/mL), KLK4 and KLK8 (each 50 nM). Fluorescence emission was measured at 535 nm. The PAR1- and PAR4-peptides are cleaved efficiently by thrombin, trypsin and KLK4. In contrast, the PAR2-peptide seems to be activated only by trypsin and KLK4, but not by KLK8 or thrombin, whereas PAR4-peptide was cleaved by trypsin, thrombin and KLK4, but not by KLK8.

V.10 Effects of KLK4, 8 and 15 on pro-uPA

In order to search further for putative KLK substrates the interplay of KLKs and the plasminogen-activation system, an important proteolytic system was analyzed. As it is known that some trypsin-like proteases process plasminogen as well as pro-uPA (Beaufort et al., 2006; Bacchiocchi et al., 2008), KLKs appeared to be good candidates. To investigate whether KLKs affect pro-uPA, the protease precursor was incubated with KLK4, KLK8 or KLK15 for different time periods with a ratio of pro-uPA to KLK corresponding to a molar ratio of 100:1. The reaction was incubated at both 37°C and room temperature. As a negative control, pro-uPA alone was treated under the same conditions. Results were assessed by SDS-PAGE (**fig.V.20**). Active KLK4 cleaves the precursor single chain peptide pro-uPA (45 kDa), which leads to active, two chain uPA, also known as HMW-uPA (17 and 30 kDa). After a time period of 1 h, the 45 kDa band of pro-uPA vanishes and a 17 and a 30 kDa band appears, which is most certainly two chain uPA. These findings might indicate that KLK4 is an optimal activator of pro-uPA and therefore could also affect the plasminogen-activation system. Interestingly, KLK8 does seem to cleave pro-uPA, but maybe not as effectively as KLK4 (**fig. V.20 a, b and V.21 b, c**). Incubation of pro-uPA with KLK8 for 1 h leads to

a weaker signal of pro-uPA (45 kDa band), but does not yield the two chain uPA. Only after 18 h two weak 17 and 30 kDa bands appear, most certainly two chain uPA. However, a weak signal for pro-uPA is still detectable. Hence KLK8 does not appear to be an optimal activator of pro-uPA. Refolded and activated KLK15 fl that was incubated with pro-uPA does not seem to activate the single precursor, as there no bands of HMW-uPA appear (**fig. V.20 c and V.21 d**). Certainly, this result may be due to the fact that KLK15 fl could not be produced in an active form, as suggested by the fluorogenic substrate assays. It is worth noting that after the reaction of KLK15 fl with pro-uPA, the concentration of the precursor seems to decrease in comparison with the starting material (0 h) and after incubation of the protein without KLK15 fl. Pro-uPA that was incubated on its own at 37°C for 22 h does not lead to auto-activation (**fig. V.20 a, b, c** samples marked with -), even so the signal of the peptide was weaker in comparison to the fresh pro-uPA sample (0 h). This might be due to slight degradation of the pro-form at 37°C.



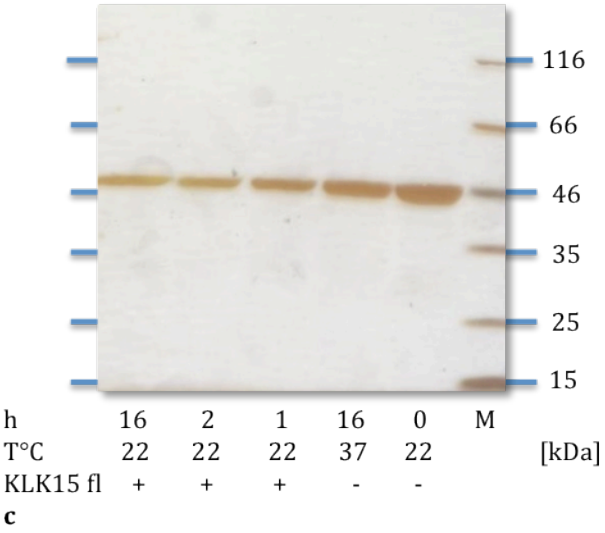


Figure V.20 Cleavage of pro-uPA by KLK4, 8 and 15 fl for different time periods. Active KLK4 in fig.a cleaves pro-uPA (45 kDa), leading to active, two chain uPA, also known as HMW-uPA (17 and 30 kDa). KLK8 in fig.b also cleaves pro-uPA, but not as effectively as KLK4. Refolded KLK 15 fl does not cleave pro-uPA. Pro-uPA has a molecular weight of about 45 kDa, cleavage by KLK4 and KLK8 gives two bands at around 15 and 30 kDa. Samples marked with (-) contained pro-uPA alone. Proteins were separated by 12% SDS-PAGE and subsequently silver-stained.

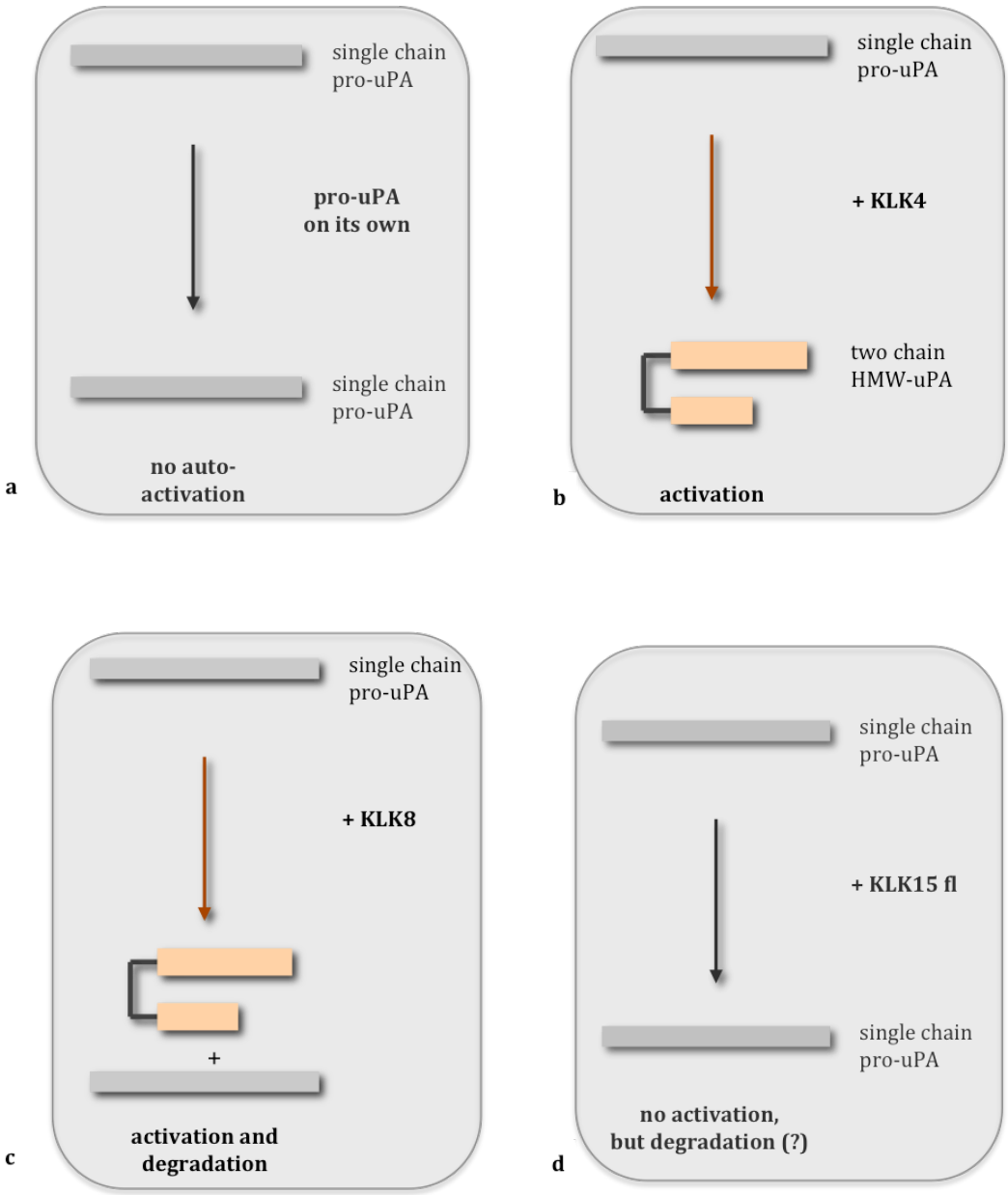


Figure V.21 Summary of the pro-uPA assay with KLK4, KLK8 and KLK15 fl. It was found that incubation of the precursor single chain peptide (45 kDa) on its own in fig.a does not result in active two chain uPA (fig.a). KLK4 in fig.b was shown to be a strong activator of pro-uPA, as the precursor is entirely cleaved even after 1 h of incubation, which leads to active HMW-uPA. KLK8 in fig.c seems to cleave the precursor peptide after 18 h, but pro-uPA is still detectable after this time period. KLK15 in fig.d does not cleave pro-uPA, as a 45 kDa band and no signal for HMW-uPA is visible after incubation.

VI Discussion

In 2006, 229,600 male and 197,600 female Germans were diagnosed with cancer. In the same year 112,438 male and 98,492 female Germans died from a tumor disease. Thus, malignant tumors are serious, life-threatening diseases . The development of cancer is a complex process that includes changes in gene structure and expression. Malignancies evolve not only from a single cause but also from interaction of various risk factors (RKI, Cancer in Germany, 2010). Some cancer types, like ovarian cancer, lack early symptoms and signs and are therefore recognized only in advanced stages when - in most cases - they cannot be cured. Hence, it is very worthwhile searching for tumor markers to assess patients and to detect malignancies at an early stage.

Certain proteolytic systems, including metalloproteases, serine proteases, and cysteine cathepsins, are involved in cancer development. These proteases modulate extracellular matrix metabolism and promote development of malignancies by affecting growth and pro-angiogenic factors, regulation of chemokines and cytokines and processing of cell-cell and cell-matrix adhesion molecules (Affara et al., 2009). Interestingly, it has been shown that several proteolytic systems act in cascades that contribute to a pathway. The kallikrein-related peptidases (KLKs) have been found to be aberrantly expressed in different cancer types, indicating that they are involved in development and progression of malignancies (Emami et al., 2007; Debela et al., 2006; Yousef et al., 2001). Further knowledge about the interplay of KLKs among themselves, other proteases or the surrounding environment, like the extracellular matrix, could help us to gain more insight in to tumor cell development. This could also lead to the development of suitable drugs that specifically target the proteases as new cancer therapeutics. KLKs can be up- or downregulated in certain malignant tumors, revealing them as potential biomarkers. An individual KLK lacks sufficient specificity and sensitivity, but might be useful in groups of KLKs or in combination with other biomarkers (Emami et al., 2008). Some KLKs indicate poor prognosis, such as KLK4 in breast, ovarian and prostate cancer (Yoon et al., 2009), others, like KLK10 allude to either favorable or unfavorable prognosis depending on the tumor type (good prognosis for breast cancer, poor prognosis for ovarian cancer).

Ovarian cancer is the second most common cancer of the female reproductive system in Germany, with 8000 women diagnosed every year. The disease does not present with early, specific symptoms. It is associated with a high mortality and poor prognosis (Kiechle et al.,

2006). Most patients are diagnosed in a stage of advanced disease, most frequently women present with FIGO stage III (Dorn et al., 2007), when the cancer has already spread to the peritoneum outside the pelvis, to the small bowel or omentum, making treatment difficult. Some biomarkers that are used in routine clinical diagnostic assays exist, such as CA-125, particularly for follow-up and monitoring of treated patients. There is growing evidence that KLKs play an important role in ovarian cancer, as KLK4, 5, 6, 7, 8, 10, 11, 13, 14, and 15 have been shown to be overexpressed in this disease (Paliouras et al., 2003). Especially KLK4 and KLK5 mRNA were found to be highly expressed, indicating unfavorable prognosis in low-grade tumors and their association with more aggressive forms of the tumor. KLK6 has been shown to be overexpressed in ovarian malignancies compared to normal epithelia. Patients with high serum levels of KLK6 were not responsive to chemotherapies and presented with a shorter disease-free and overall survival. Interestingly, the combination of KLK6 with CA-125 leads to a higher sensitivity and specificity in detection and monitoring of ovarian cancer, compared to CA-125 alone (Paliouras et al., 2003). Overexpression of KLK10, a favorable marker for breast cancer, is associated with serous histotype, advanced stage, and large residual tumor size in ovarian carcinoma, suggesting KLK10 as a new serological and tissue marker for diagnosis, monitoring and prognosis of the disease. It is worth noting that expression of some members of the kallikrein family, such as KLK11 and 13, indicate a better outcome for overall survival in malignant ovarian tumors (Paliouras et al., 2003). KLK11 and 13 positive tumors were associated with early stage (FIGO I and II) and better response to chemotherapies (Paliouras et al., 2003). Development of more sensitive ELISAs and other, multiparamic analysis of tissues and biological fluids would help to diagnose patients in earlier stages and to better predict prognosis, response to therapy and outcome.

VI.1 KLKs interplay with cancer-related targets

KLK15 mRNA is detectable at higher levels in ovarian cancer samples compared to benign ovarian tissue (Mavridis et al., 2010). A novel splice variant, called KLK15 ntfl (meaning near to full length), was found by PCR analysis in ovarian cancer specimens in the laboratory of the Clinical Research Unit (Department of Gynecology, Technical University of Munich). It is lacking 14 amino acids compared to full length KLK15 (KLK15 fl). Unfortunately, it was not possible to produce significant amounts of either active KLK15 fl or KLK15 ntfl. A rapid

degradation during the enterokinase-induced activation step of KLK15 fl and 15 ntfl was observed, indicating that either KLK15 is an excellent target for enterokinase and digested too efficiently or that KLK15 is potentially auto-proteolytic or auto-catalytic subsequent to its activation by enterokinase. To further investigate KLK15, an inactive KLK15 mutant (KLK15 S/A) was produced using *in vitro* site-directed mutagenesis, which generated an active site serine to alanine mutant of KLK15 fl. Purified and folded KLK15 S/A incubated with enterokinase was found to be degraded, resulting in a decreasing signal on SDS-PAGE likewise to KLK15 fl and 15 ntfl, suggesting that KLK15 S/A is a good target for enterokinase. On the basis of this result, we conclude that the rapid degradation of wild-type KLK15 and variants thereof by enterokinase are mainly due to enterokinase are not mediated by self-degradation.

Although the recombinant proteases were found to be degraded, we were able to generate enough cleaved protein, which had the expected molecular mass of the mature enzyme. N-terminal sequencing, performed on the cleaved KLKs by the lab of Dr. J. Kellermann and Prof. Dr. F. Lottspeich at the Max-Planck-Institute for Biochemistry, Martinsried, Germany (section Protein Analysis), verified that incubation of the KLKs leads to the formation of the mature N-terminus of KLK15 fl, 15 ntfl and 15 S/A (as well as KLK4 and 8), indicating the active form of the protease. These findings suggest that activated, mature KLK15 might not be correctly folded, therefore lacks activity and cannot cleave substrates. Further research is needed to establish how enterokinase acts specifically on KLK15 fl and 15 ntfl. The production of recombinant active KLK4 and 8 was successful. Results verified that KLK4 and 8 are trypsin-like serine proteases, preferring arginine or lysine at P_1 (Beaufort et al., 2006). Using active KLKs, the interactions of those proteases with potential cancer-related targets namely the plasminogen-activation system and the protease-activated receptors were explored.

VI.2.1 Proteases and cancer-related substrates

VI.2.1.1 PARs

Recently, it has been shown that serine proteases can act as signaling molecules by cleaving and triggering members of proteinase-activated receptors (PARs) (Hollenberg et al., 2008; Ramsay et al., 2008; Yoon et al., 2007; Beaufort et al., 2006), specific transmembrane receptor, that control cell functions. PARs activation results in cytosolic calcium mobilization and intracellular signaling, followed by a rapid and transient desensitization of the receptor via endocytosis. PARs were found to play a role in many pathologies such as cancer, arthritis, asthma, inflammatory bowel disease, neurodegeneration and cardiovascular disease (Mize et al., 2008; Darmoul et al., 2003). Cleavage of protease-activated receptors by KLK4 and KLK8 was evaluated using seven-residue peptides that represent cleavage sites in PAR1, 2 and 4. Results showed that the PAR1-peptide is cleaved efficiently by thrombin, trypsin and KLK4, whereas the PAR2-peptide was cleaved only by trypsin and KLK4, but not by KLK8 or thrombin. The PAR4-peptide is cleaved by trypsin, thrombin and KLK4, but not by KLK8.

VI.2.1.2 Involvement of PARs in colon cancer

Further studies of our group and collaborators showed that KLK4 by cleaving PAR1 might be involved in human colon cancer cell proliferation, see **fig.VI.1** (Gratio et al., 2009). Immunohistochemical analysis revealed that KLK4 is highly expressed in human colon adenocarcinomas in contrast to its low level expression in normal epithelia. Using a colon cancer cell model, KLK4 was found to initiate a loss of PAR 1 and PAR 2 from HT29 cell surface, leading to Ca^{2+}-flux, which can be abrogated after initial challenge with TRAP (a molecule that desensitizes PAR 1 and PAR 2). An antibody blocking PAR 1 reduced KLK4-induced Ca^{2+} influx, whereas a PAR 2-blocking antibody only slightly affected the KLK4-induced Ca^{2+} flux, suggesting that KLK4 might activate PAR 1 but not PAR 2 in HT29 cells. Moreover, it was verified that KLK4 induces intracellular signaling by rapid and significant Erk1/2 phosphorylation in HT29 cells. Darmoul et al. observed that PAR 1 could function as an oncoprotein, transforming fibroblasts *in vitro*, suggesting that the activation of PAR 1 plays an important role in the development of human colon cancer (Darmoul et al., 2003). Taking this into consideration, it appears that KLK4, as it is highly expressed in colon cancer, might lead to colon cancer cell proliferation mediated through PAR 1.

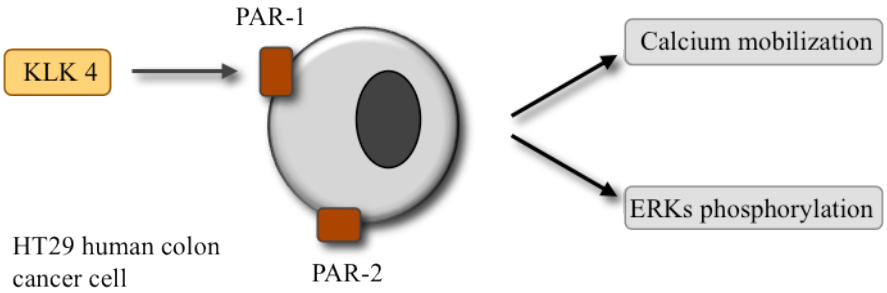


Figure VI.1 KLK4 activates PAR 1 in HT29 human colon cancer cells. PAR1 activation leads to intracellular calcium mobilization and phosphorylation of ERKs. These findings suggest that KLK4 may play a role in the development of cancer cells. Taken and modified from Gratio et al., 2009.

VI.2.1.3 Plasminogen activation system mediates cellular events

Another proteolytic system known to be involved in development of malignancies is the plasminogen activation system, which is composed among others of the urokinase-type plasminogen activator (uPA), its receptor (uPAR) and plasminogen (**fig. VI.2**). This system plays an important role in cancer invasion, due to the capability of uPAR to concentrate and increase the proteolytic activity of uPA on the cell surface. uPAR was found at the migration front in active, moving cells, thus focusing the proteolytic activity of uPA where it is needed. The receptor, a multifunctional protein, is involved in proteolytic and non-proteolytic processes including cell adhesion, migration, chemotaxis and tissue remodeling. High expression levels of uPAR in various human malignancies, such as colon, breast, ovarian, stomach and lung cancer were found to be correlated with unfavorable prognosis, indicating that the receptor promotes progression and aggressiveness of cancer cells (Bacchiocchi et al., 2008). In many types of solid cancer, including epithelial ovarian cancer, uPA is associated with progression and poor prognosis of the tumor. A recent study revealed that uPA and uPAR are overexpressed in primary and metastatic ovarian tumors. Immunohistochemistry showed that uPA and uPAR were expressed mostly by tumor and stromal cells. Positive staining was found to be significantly associated with tumor stage, grade and time to relapse, indicating an unfavorable prognosis and reduced progression-free survival (Dorn et al., 2007). Other proteases, such as MMPs, were co-localized with uPA (Schilling et al., 2008), suggesting interactions that promote development and growth of tumor cells. There is evidence that KLKs interact with the plasminogen-activation system, as these proteases are known to be involved in wound healing mediated through plasmin (Castellino et al., 2006). Using an *in vitro* system, the protease zymogen pro-uPA was

identified as a cancer-related target of KLKs, it was found to be activated by KLK4, in contrast to plasminogen, which was left inactive. There is evidence that KLK4 acts as a key modulator of the uPA-uPAR system; the protease can activate tumor-associated proteolysis by activation of pro-uPA and affects tumor cell behavior by cleaving uPAR (Beaufort et al., 2006). Moreover, uPAR can concentrate and increase uPA's proteolytic activity on the cell surface, amplifying the effect of KLK4. In the present work, KLK8 was found to activate pro-uPA, albeit to a lesser extent.

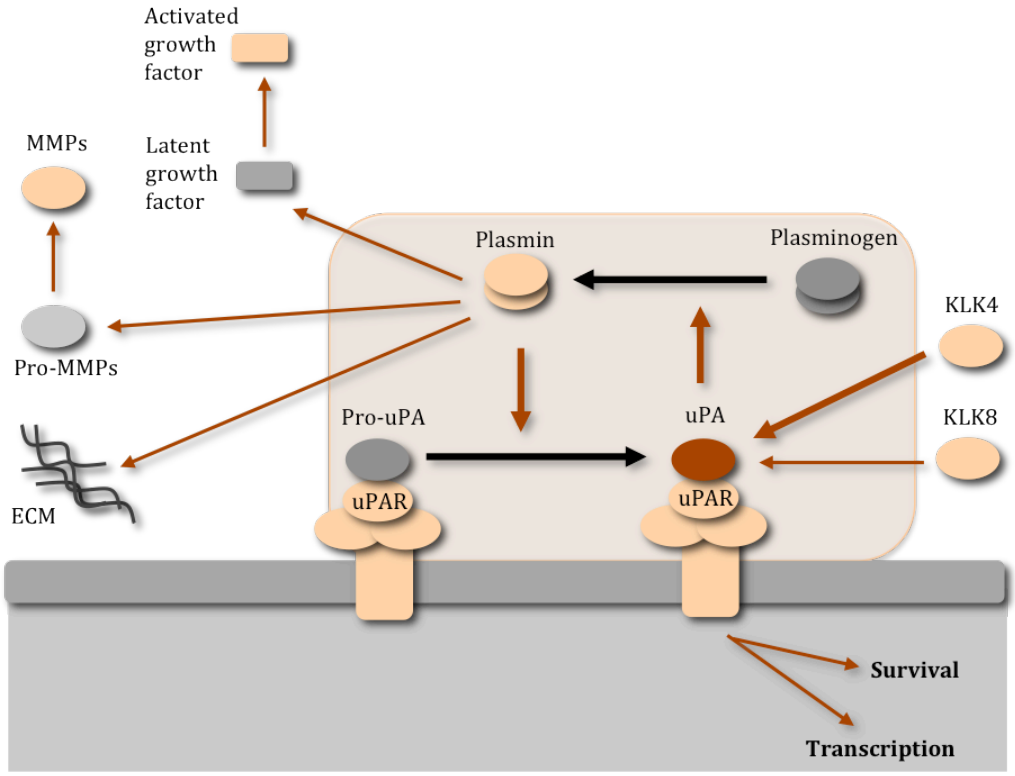


Figure VI.2 The urokinase-type plasminogen activator receptor (uPAR) mediates cellular events on the cell surface. Binding of pro-uPA to uPAR provides the cell surface with potential plasmin-dependent proteolytic activity that determines matrix degradation. This activates growth factors and proteases such as MMPs. Furthermore, by activating uPAR, signaling pathways are generated affecting survival and transcription of proteins. KLK4 and 8 may thus be involved in several signaling pathways by activating pro-uPA. ECM: extracellular matrix. Taken and modified from Rao et al., 2003.

VI.2.2 Recent work and research results: KLK15

In 2001, Takayama et al. first described prostinogen, a serine protease that is highly expressed in the prostate gland that was later named KLK15. They suggested that the protease was a possible candidate for activation of proKLK3 (Pamplakis et al., 2007). cDNA of KLK15

was inserted behind the initiator methionine codon of a pET12 vector and expressed in *E. coli* (BL21(DE3) strain). Solubilized inclusion bodies were purified using benzamidine, subsequently diluted and flushed under nitrogen. Refolded KLK15 was activated with trypsin-agarose and activity was assessed using the chromogenic substrate S-2222 (Takayama et al., 2001), see **fig. VI.3**. It is worth noting that this group succeeded in producing active KLK15. This might be due to a variety of reasons, such as a better construct, more effective expression methods/facilities, activation techniques with trypsin-agarose or just a more suitable substrate for KLK15 (S-2222). However, it should be noted that activity could be also simulated by trypsin that was not removed efficiently from the solution.

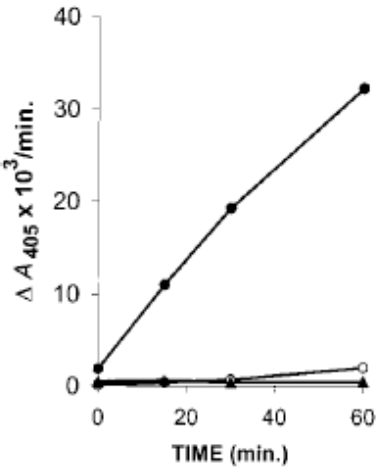


Figure VI.3: KLK15 activation with trypsin agarose. Unpurified KLK15 (190 µg) was activated with trypsin-agarose (5 µL) in 110 µL total volume. The reaction was terminated at various times by centrifugation of 25 µL aliquots. Enzyme activity of 15 µL of the samples was measured using the substrate S-2222 (0.5 mM final). Activated prostin (black circles) showed substantial amidolytic activity, while KLK15 (triangles) and the control (sample without KLK15; open circles) did not show any activity. Taken from Takayama et al., 2001.

Latest studies from Yoon et al. (2009) showed that KLK15 has significant activity towards both Arg- and Lys-containing KLK pro-sequences. KLK15 was found to activate proKLK8 and 14. These results suggest that active KLK15 can potentially communicate with additional proKLK signaling cascades. Interestingly, KLK15 displayed highest efficiency in hydrolyzing its own pro-peptide sequence, suggesting auto-catalytic activity. Furthermore it was shown, that KLK11 is able to hydrolyze proKLK15 (hydrolysis after the P_1 leucine residue), indicating KLK11 as a possible activator of proKLK15 (Yoon et al., 2007). Moreover, Yoon et al. observed that hydrolysis of proKLK15 by KLK4 results in a permanently inactive form of KLK15.

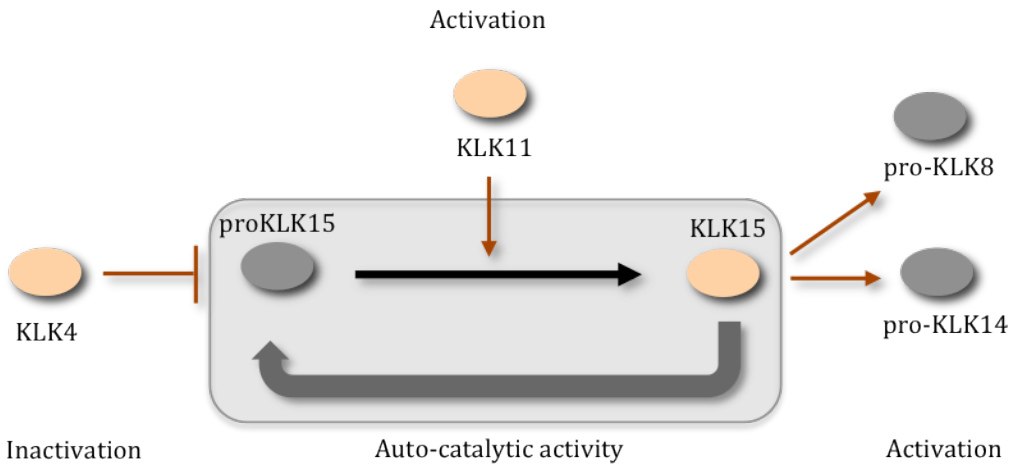


Figure VI.4 KLK15 interplay with other KLKs. ProKLK was shown by other groups to be activated by KLK11. KLK15 can then cleave proKLK8 and 14, which will activate the proteases. Interestingly, KLK15 was found to have a strong auto-catalytic activity towards its own pro-form, which leads to a high amount of inactivated protein. Furthermore KLK4 was confirmed to inhibit the activation of proKLK15. Taken from Yoon et al, 2007; Yoon et al., 2009.

VI.3 Finding possible substrates of KLKs: extended substrate specificity

For further analysis of KLKs, different techniques are available to assess interactions of KLKs with other proteases and possible substrates. Extended substrate specificity profiles may help in finding their physiological protein substrates as well as designing more selective inhibitors and lead to a better understanding of human tissue kallikrein functions. One technique uses positional scanning of the P_1-P_4 positions with fluorogenic peptide substrates from a synthetic combinatorial library, called PS-SCL screening (Debela et al., 2006). For each P_1-P_4 position the substrate cleavage rates in picomolar concentrations of fluorophore released per sec are assessed, as shown in **figure VI.5** for KLK4. Results demonstrate that KLK4 exhibits trypsin-like activity, with a strong preference for arginine at $P_{1'}$. Using PS-SCL screening techniques, it is possible to analyze minor structural and functional differences of KLKs that might be essential in order to understand their special physiological function.

The positional scanning data corresponds to structural data and may be useful when searching for physiologically relevant substrates. It should also be taken into consideration that the amino acid preferences at P_1-P_4 positions from the scanning data excludes certain contributing effects between the four side chains, particularly between P_2 and P_4 (Debela et al., 2006).

Recently, a technique for subsite specificity profiling called PICS (proteomic identification of

cleavage sites) was developed in the Overall Laboratory, UBC, Vancouver, Canada. The same laboratory developed another technique, called TAILS, to screen for the native substrates of proteases.

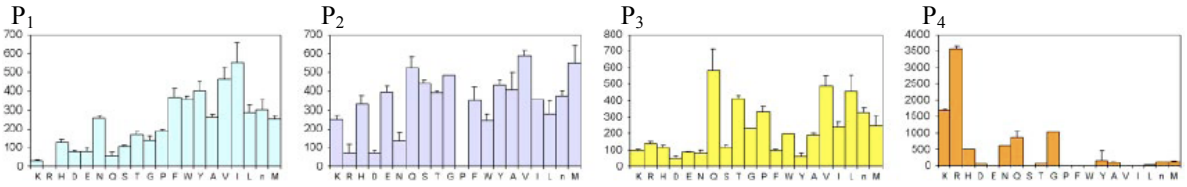


Figure VI.5: Extended substrate specificity of KLK4. Results from P_1, P_2, P_3, and P_4 diverse positional scanning library for recombinant mature KLK4; *y-axis* represents the substrate cleavage rates in picomolar concentrations of fluorophore released per sec. The *x-axis* indicates the amino acid held constant at each position, designated by the one-letter code (with *n* representing norleucine). Taken from Debela et al., 2006.

VI.4 Future directions

The present work focused on the expression of recombinant human KLK4, 8, and 15 in *E. coli* to allow biochemical characterization identification of substrates of these enzymes.

In the case of KLK4, the X-ray structure of the enzyme has been resolved (Debela et al., 2008), a substrate profiling performed (Debela et al., 2008) and possibly tumor-relevant substrates such as members of the plasminogen activation system, pro-uPA and uPAR, identified (Beaufort et al. 2006; 2009). Furthermore, based on initial findings using PAR-derived peptides (this work), we were able to demonstrate that, in colon cancer cells, KLK4 targets and activates PAR1, which may in fact represent a novel pathway in colon tumorigenesis. To evaluate interaction of KLK4 with the tumor environment in more detail, proteome-wide screening methods such as TAILS should be conducted in order to identify native, physiological substrates of the protease. Moreover, using the structural data as well as analytical data obtained from extended subsite specificity and substrate screening methods, specific inhibitors could be developed to target KLK4 for therapeutic inhibition, e.g. in ovarian cancer.

As demonstrated in the present work, KLK8 can also be recombinantly produced in *E. coli* yielding an active, mature activation enzyme by enterokinase. Meanwhile, the X-ray structure of KLK8 has been resolved and also substrate profiling studies performed (M. Debela, V. Magdolen, P. Goettig, pers. comm.). Thus, similarly to KLK4, other relevant substrates

maybe identified with TAILS and PICS (Schilling et al., 2008; 2007). New data point in particular to an important function of KLK8 within the human epidermis. Using keratinocytes as the cellular system for identification of natural substrates of KLK8 may shed light on its role in the skin.

In contrast to KLK4 and KLK8, KLK15 was not successfully isolated as an active protease using the *E. coli* expression system. For successful production of active KLK15, different alternative methods and expression systems should be tested (bacterial, insect cells with baculoviruses and mammalian cells), which might then allow progress with biochemical and structural characterization of this enzyme in a similar manner to KLK4 and KLK8.

In addition to the basic research, it is certainly necessary to perform more clinical studies with cancer patients, quantify KLKs and possible substrates in tumor samples on the mRNA or antigen level and correlate results with clinical outcome and other features, such as response to chemotherapy. This would help identify groups of KLKs and substrates of KLKs as new clinical biomarkers.

VII Summary

Human tissue kallikreins, also known as kallikrein-related peptidases (KLKs), are a subgroup of extracellular serine proteases that belong to the S1 family of the clan SA. They are expressed in various tissues and cell types, implicating a wide range of normal physiological functions. Interestingly, these proteases display aberrant expression patterns in a number of cancer types, for which their substrates and functions are still poorly characterized. Certain KLKs have been found to be overexpressed, as well as downregulated in different cancer types and are thus are suspected of playing a role in the development of malignancies. Many KLKs have therefore been identified as promising diagnostic/prognostic biomarkers for several cancer types such as ovarian, breast and prostate cancer. To evaluate the role of selected members of the KLK family, especially in the context of disease and malignancies, the laboratory of the Clinical Research Unit (TUM) aims to produce active KLKs in order to allow biochemical and structural characterization and identification of tumor-relevant substrates of these enzymes.

The present thesis focused on the expression and purification of three KLKs: KLK4, KLK8, and KLK15. All three KLKs were recombinantly produced in *E. coli* as synthetic pro-enzyme forms. The synthetic pro-peptide harbored a histidine$_6$-tag, allowing purifictation by affinity chromatography via Ni^{2+}-NTA-agarose, followed by an optimal enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys↓). The recombinant proteins were purified under denaturing and reducing conditions and thus had to be refolded prior to activation by enterokinase.

In the case of KLK4 and KLK8, active, mature enzymes were generated, as the proteases were shown to efficiently cleave small fluorgenic peptide substrates. Although the pro-peptide of recombinant KLK15 - by N-terminal protein sequencing - was shown to be correctly cleaved off by enterokinase, the mature enzyme did not cleave any of the analyzed substrates. In addition to wild-type KLK15 (also known as KLK15 full length, KLK15 fl), a splice variant of KLK15 (KLK15 ntfl; "near to full length") was investigated, which has been previously found to be expressed in ovarian cancer. Similar to KLK15fl, no activity was observed for KLK15 ntfl after correct removal of the synthetic pro-peptide. Incubation of both KLK15fl and KLK15 ntfl with enterokinase results in a significant degradation of the recombinant proteins. To investigate whether this degradation is due to cleavage of the recombinant proteins by enterokinase or to self-degradation of the KLK15 proteins after activation by enterokinase, an active site serine to alanine mutant of KLK15 fl (KLK15-S/A) was generated by *in vitro* mutagenesis in order to produce an inactive KLK15 mutant.

As KLK15-S/A was also prone to degradation after incubation with enterokinase, it can be concluded that enterokinase efficiently degrades KLK15.

Using the recombinant active enzymes, interactions of KLK4 and 8 with the pro-enzyme form of the serine protease uPA, a member of the plasminogen activation system was analyzed. Furthermore, the cleavage patterns of both enzymes towards peptides covering the activation site of proteinase-activated receptor (PAR) 1, 2 and 4 were explored. KLK4 was found to efficiently activate pro-uPA and cleave all PAR-derived peptides. Interestingly, in subsequent cellular analysis, KLK4 was found to specifically activate only PAR1 in colon cancer cells. KLK8 appeared to be rather a weak activator of pro-uPA, but did not cleave any of the PAR-peptides.

In the future, alternative expression systems for wild-type KLK15 and its variant KLK15-ntfl, such as insect, rodent or human cells, should be considered in order to achieve biochemical and structural characterization of these proteins. In case of KLK4 and KLK8, the recombinant enzymes can be used for further analyses to identify other natural substrates which could unmask the pathophysiological roles of these enzymes. Moreover, structural and also biochemical information, such as substrate specificity, may be the basis for the development of specific inhibitors targeting KLKs thus helping to establish novel KLK-directed therapies in cancer treatment.

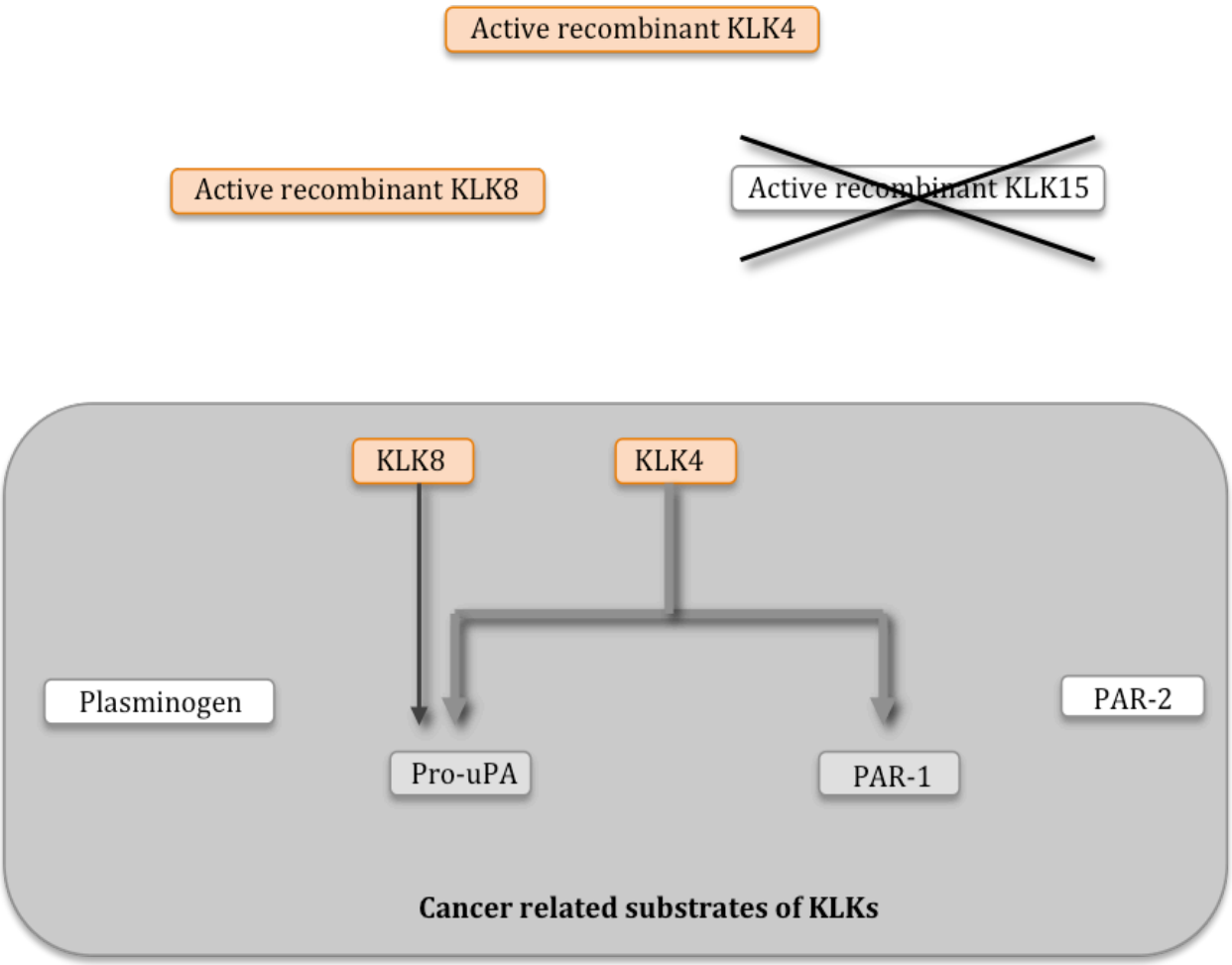


Fig.VI.6 Summary of the present thesis. Using a prokaryotic protein expression system, active recombinant human KLK4 and KLK8, but not KLK15 was produced. Cancer-related targets for KLK4 including pro-uPA and PAR-1 were identified. KLK8 was found to activate pro-uPA.

VIII References and literature

Affara N I, Andreu P, Coussens L M (2009) Delineating protease functions during cancer development. Methods of Molecular Biology, 539, 1-32

Bacchiocchi R, Rubini C, Pierpaoli E, Borghetti G, Procacci P, Nocini P F, Santarelli A, Rocchetti R, Ciavarella D, Lo Muzio L, Fazioli F (2008) Prognostic value analysis of urokinase-type plasminogen activator receptor in oral squamous cell carcinoma: an immunohistochemical study. Biomed Center Cancer, 8, 220

Beaufort N, Debela M, Creutzburg S, Kellermann J, Bode W, Schmitt M, Pidard D, Magdolen W (2006) Interplay of human tissue kallikrein 4 (hk4) with plasminogen activation system: hk4 regulates the structure and functions of the urokinase-type plasminogen activator receptor (uPAR). Journal of Biological Chemistry, 387, 217-222

Berg J M, Tymoczko J L, Stryer L (2002) Biochemistry. W H Freeman and Co., fith edition

Beynon R J, Bond J S (2001) Proteolytic Enzymes: A Practical Approach, second edition. Oxfort University Press

Böcker W, Denk H, Heitz P U (2004) Pathologie, Urban & Fischer, Chapter 6: Allgemeine Tumorpathologie. 169 – 223

Borgoño C A, Michael I P, Diamandis E P (2004) Human tissue kallikreins: physiologic roles and applications in cancer. Molecular Cancer Research, 2, 257-280

Cancer in Germany 2005 – 2006. Incidence and Trends (2010) Robert Koch Institute and the Association of Population-based Cancer Registries in Germany, Seventh edition

Cane S, Bignotti E, Bellone S, Palmieri M, De Las Casas L, Roman J J, Pecorelli S, Cannon M J, O'Brien T, Santin A D (2004) The novel serine protease tumor-associated differentially expressed gene-14 (KLK8/Neuropsin/Ovasin) is highly overexpressed in cervical cancer. American Journal of Obstetrics and Gynecology, 190, 60-66

Castellino F J, Ploplis V A (2005) Structure and function of the plasminogen/plasmin system. Journal of Thrombosis and Haemostasis, 93, 647-654

Clements J A (2008) Reflections on the tissue kallikrein and kallikrein-related peptidase family – from mice to men – what have we learnt in the last two decades? Journal of Biological Chemistry, 389, 1447–1454

Darmoul D, Gratio V, Devaud H, Lehy T, Laburthe M, (2003) Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. American Journal of Pathology, 162, 1503-1513

Davie E W, Fujikawa K, Kisiel W (1991) The coagulation cascade: initiation, maintenance, and regulation. Biochemistry, 30, 10363-10370

Debela M, Beaufort N, Magdolen V, Schechter N M, Craik C S, Schmitt M, Bode W, Goettig P (2008) Structures and specificity of the human kallikrein-related peptidases KLK4, 5, 6, and 7. Journal of Biological Chemistry, 389, 623-632

Debela M, Magdolen V, Grimminger V, Sommerhoff C, Messerschmidt A, Huber R, Friedrich R, Bode W, Goettig P (2006) Crystal structures of human tissue kallikrein 4: activity modulation by a specific zinc binding site. Journal of Molecular Biology, 362, 1094–1107

Debela M, Magdolen V, Schechter N, Valachova M, Lottspeich F, Craik C S, Choe Y, Bode W, Goettig P (2006) specificity profiling of seven human tissue kallikreins reveals Individual subsite preferences. Journal of Biological Chemistry, 281, 25678-25688

Didenko V V, Editor (2006) Fluorescent energy transfer nucleic acid probes: designs and protocols (Methods in Molecular Biology), 1 edition. Humana Press, 17-29

Dorn J, Schmitt M, Kates R, Schmalfeldt B, Kiechle M, Scorilas A, Diamandis E P, Harbeck N (2007) Primary tumor levels of human tissue kallikreins affect surgical success and survival in ovarian cancer patients. Clinical Cancer Resaerch, 13, 1742-1748

Emami N, Diamandis E P (2007) Human tissue kallikreins: a road under construction. Clinica Chimica Acta, 381, 78-84

Emami N, Diamandis E P (2008) Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. Clinical Chemistry, 54, 1600-1607

Erez E, Fass D, Bibi E (2009) How intramembrane proteases bury hydrolic reactions in the membrane. Nature, 459, 371-378

Goettig P, Magdolen V, Brandstetter H (2010) Natural and synthetic inihibitors of kallikrein-related peptidases (KLKs), Biochimie, 92, 1546-1567

Goettig P, Magdolen V, Brandstetter H, Gratio V, Beaufort N, Seiz L, Maier J, Virca G D, Debela M, Grebenchtchikov N, Magdolen V, Darmoul D (2009) Kallikrein-related peptidase 4 (KLK4): a new activator of the aberrantly expressed protease-activated receptor 1 in colon cancer cells. American Journal of Pathology, 176, 1452-61

Hollenberg M D, Oikonomopoulou K, Hansen K K, Saifeddine M, Rithwik R, Diamandis E P (2008) Kallikreins and proteinase-mediated signaling: proteinase-activated receptors (PARs) and the pathophysiology of inflammatory diseases and cancer. Journal of Biological Chemistry, 389, 643–651

Kiechle M (2006) Gynäkologie und Geburtshilfe. Urban & Fischer, Elsevier, 1. Auflage

Kishi T, Cloutier S M, Kuendig C, Deperthes D, Diamandis E P (2006) Activation and enzymatic characterization of recombinant human kallikrein 8. Journal of Biological Chemistry, 387, 723–731

Kuhn W, Schmalfeldt B, Reuning U, Pache1 L, Berger U, Ulm K, Harbeck N, Späthe K, Dettmar P, Höfler H, Jänicke F, Schmitt M, Graeff H (1999) Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. British Journal of Cancer, 79, 1746–1751

Light A, Janska H (1989) Enterokinase (enteropeptidase): comparative aspects. Biochemical Science, 14, 110-112

Mann N S, Mann S K (1994) Enterokinase. Proceedings of the society for experimental biology and medicine, 206, 114-118

Martin L P, Schilder R J (2009) Management of recurrent ovarian carcinoma: current status and future directions. Seminars in Oncology, 36, 112-125

Mavridis K, Scorilas A (2010) Prognostic value and biological role of the kallikrein-related peptidases in human malignancies. Future Oncology, 6, 269-285

Mize G J, Wang W, Takayama T K (2008) Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2. Molecular Cancer Research, 6, 1043-1051

Murray R K, Granner D K, Mayes P A, Rodwell V W (2006) Harper's Illustrated Biochemistry (Harper's Biochemistry), 27 edition, McGraw-Hill Medical. Section I: Structures & Functions of Proteins & Enzymes, Chapter 5: Proteins: Higher Orders of Structure

Paliouras M, Borgono C, Diamandis E P (2007) Human tissue kallikreins: the cancer biomarker family. Cancer Letters, 249, 61-79

Pamplakis G, Sotiropoulou G (2007) Tissue kallkirein Proteolytic cascade pathways in normal physiology and cancer. Biochimica et Biophysica Acta, 1776, 22-31

Pearce C L, Near A M, Van Den Berg D J,(2009) Validating genetic risk associations for ovarian cancer through the international Ovarian Cancer Association Consortium. British Journal of Cancer, 100, 412-420.

Petsko G A, Ringe D (2003) Protein Structure and Function. 1 edition, New Science Press, Ltd.

Prezas P, Arlt M J, Viktorov P, Soosaipillai A, Holzscheiter L, Schmitt M, Talieri M, Diamandis E P, Krüger A, Magdolen V (2006) Overexpression of the human tissue kallikrein genes KLK4, 5, 6, and 7 increases the malignant phenotype of ovarian cancer cells. Journal of Biological Chemistry, 387, 807-811

Qiagen (2003) The QIAexpressionist™: A handbook for high-level expression and purification of 6xhis-tagged proteins. fith edition

Qiagen (2006) QIAprep Miniprep® Handbook, second edition

Ramsay A J, Reid J C, Adams M N, Samaratunga H, Dong Y, Clements J A, Hooper J D (2008) Prostatic trypsin-like kallikrein-related peptidases (KLKs) and other prostate expressed tryptic proteinases as regulators of signaling via proteinase-activated receptors (PARs). Journal of Biological Chemistry, 389, 653-668

Rao J S (2003) Molecular mechanisms of glioma invasiveness: the role of proteases. Nature Reviw Cancer, 3, 489-501

Reynard J, Brewster S, Biers S (2005) Oxford Handbook of Urology. Oxford University Press, Chapter 3: Urological investigations, 45-67, Chapter 7: Urological neoplasia, 189-323

Schilling O, Overall C M (2007) Proteomic discovery of protease substrates. Current Opinion in Chemical Biology, 11, 36-45

Schilling O, Overall C M (2008) Proteome-derived, database-searchable peptide libraries for identifying protease cleavage sites. Nature Biotechnology, 26, 685-694

Schmalfeldt B, Prechtel D, Harting K, Spathe K, Rutke S, Konik E, Fridman R, Berger U, Schmitt M, Kuhn W, Lengyel E (2001) Increased expression of matrix metalloproteinases (MMP)-2, MMP-9, and the urokinase-type plasminogen activator is associated with progression from benign to advanced ovarian cancer. Clinical Cancer Research, 2396, 2396–2404

Shaw J, Grass L, Sotiropoulou G, Diamandis E P (2006) Development of an immunofluorometric assay for human kallkrein 15 (KLK15) and identification of KLK15 in tissues and biological fluids. Clincal Biochemistry, 40, 104-110

Takayama T K, Carter C A, Deng T (2001) Activation of prostate-specific antigen precursor (pro-PSA) by prostin, a novel human prostatic serine protease identified by degenerate PCR. Biochemistry, 40, 1679-1687

Vu T K, Hung D T, Wheaton V I, Coughlin S R, (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell, 64, 1057-1068

Wang L, Madigan M C, Chen H, Liu F, Patterson K I, Beretov J, O'Brien P M, Li Y (2009) Expression of urokinase plasminogen activator and its receptor in advanced epithelial ovarian cancer patients.Gynecology Oncology, 2009 May 16

Yamashina I (1956) The action of enterokinas on trypsinogen. Biochimica et Biophysica Acta, 20, 433-434

Yoon H, Blaber S I, Debela M, Goettig P, Scarisbrick I A, Blaber M (2009) A completed KLK activome profile: investigation of activation profiles of KLK9, 10, and 15. Journal of Biological Chemistry, 390, 373-377

Yoon H, Laxmikanthan G, Lee J, Blaber S I, Rodriguez A, Kogot J M, Scarisbrick I A, Blaber M (2007) Activation profiles and regulatory cascades of the human kallikrein-related peptidases. Journal of Biological Chemistry, 282, 31852-31864

Yousef G M, Diamandis E P (2001) The new human tissue kallikrein gene family: structure, function, and association to disease. Endocrinology Review, 22, 184-204

Yousef G M, Scorilas A, Katsaros D, Fracchioli S, Iskander L, Borgono C, de la Longrais I A R, Puopolo M, Massobrio M, Diamandis E P (2003) Prognostic value of the human kallikrein gene 15 expression in ovarian cancer. Journal of Clinical Oncology, 21, 3119-3126

Yousef G M, Scorilas A, Magklara A, Memari N, Ponzone R, Sismondi P, Biglia N, Ellatif M, Diamandis E P (2002) The androgen-regulated gene human kallikrein 15 (KLK15) is an independent and favourable prognostic marker for breast cancer. British Journal of Cancer, 87, 1294 – 1300

Yousef G M, Diamandis E P (2003) An overview of the kallikrein gene families in humans and other species: emerging candidate tumour markers. Clinical Biochemistry, 36, 443-452

Yousef G M, Scorilas A, Jung K, Ashworth L K, Diamandis E P (2001) Molecular cloning of the human kallkirein 15 gene (KLK15). Up-regulation in prostate cancer. Journal of Biological Chemistry, 276, 53-61

IX Publications and Conferences attended

Gratio V, Beaufort N, Seiz L, Maier J, Virca GD, Debela M, Grebenchtchikov N, Magdolen V, Darmoul D (2010) Kallikrein-related peptidase 4: a new activator of the aberrantly expressed protease-activated receptor 1 in colon cancer cells. Am. J. of Pathology, 176, 1452-61

Starr A, Dufour A, Maier J, Overall C (2012) Enhanced chemoattraction of monocytes following matrix metalloproteinases (MMP) activation of chemokines CCL15 and CCL23 and increased glycoaminoglycan binding of CCL16. J. of Biol. Chemistry, 287, 5848-60

Winterschool Proteases and their Inhibitors 2007 and 2008, Tier, Italy

X Curriculum vitae

PERSONAL DETAILS

Name: Josefine Theresia Maier
Date and place of birth: April 6th 1985, Berlin
Nationality: German

WORK

Jan '13 – now	Resident in Obstetrics and Gynecology, Vivantes Klinikum im Friedrichshain, Department of Obstetrics and Gynecology, Berlin
Aug '11 – Dec '12	Resident in Cardiology, Vivantes Klinikum am Urban and im Friedrichshain, Department of Cardiology, Berlin

EDUCATION

'04–Apr'11	Technical University of Munich, Germany (TUM) Study of human medicine, clinical part Grade 1 (90 out of 100)
Feb-Jul '09	Erasmus-Scholarship, Université Paul-Sabatier III, Toulouse, France, Study of human medicine within the exchange program
Sept '06	Transfer to TUM
Aug '06	First state medical examination (1st part of the preclinical examination in medicine), Ludwig-Maximilian University, Munich Grade 2 (80 out of 100)
Oct '04-Aug '06	Ludwig-Maximilian University, Munich (LMU) Study of human medicine, preclinical part, which is comparable to Undergraduate School
Jul '04	High School Certificate (Abitur - university entrance qualification), Friedrich-List-Oberschule, Berlin , Germany Grade: 1.8 (86.7 out of 100)

WORKEXPERIENCE

Apr-Jul '10	Placement as part of the "Practical Year": Internal Medicine Department of Cardiology, Deutsches Herzentrum Munich Department of Gastroenterology, Klinikum Rechts der Isar, Munich
Dec-Apr '10	Placement as part of the "Practical Year": Pediatrics Department of Pediatric Oncology, Children's Hospital Munich Department for Pediatric Rheumatology, Children's Hospital of Montreal, McGill University, Montréal, Quebec, Canada
Aug-Dec '09	Placement as part of the "Practical Year": Surgery

	Department for Pediatric Surgery, CHU St. Justine, affiliated with Université de Montréal, Quebec, Canada Department of Thoracic Surgery, St. Joseph's Hospital, affiliated with McMaster University, Hamilton, Ontario, Canada
Mai-Jul '09	Clinical Rotation Day Hospital for Diabetology, CHU Rangueil, Toulouse, France
Feb-Mai '09	Clinical Rotation Neonatology Ward, Hôpital des Enfants, CHU Purpan, Toulouse, France
Feb '09	26th Winter School: "Proteases and their Inhibitors", Tiers, Italy: I gave the talk "Production of active, recombinant, human kallikrein-related peptidases: analysis of effects on proteins-activated receptors".
Aug-Sept '08 -	Summer Student Overall Laboratory, Centre for Blood Research, University of British Columbia, Vancouver, Canada I studied the function of a cleavage product of a specific cytokine in the immune system. Supervised by a senior Ph.D. student I had the chance to learn new methods and techniques in the Laboratory such as cell culture, cell isolation, immuno assays and staining methods.
Feb '08	25th Winter School: "Proteases and their Inhibitors", Tiers, Italy
Aug '07- present	Doctoral Thesis Department of Gynecology and Obstetrics, TUM, Germany, Research group of Prof. Schmitt
Jul-Aug '07	Clinical Rotation Department of Gynecology and Obstetrics, TUM, Germany
Mar-Jul '07	Gynecological Examination Class led by PD Dr. Höß, TUM, Germany
Mar '07	Clinical Rotation Practice for Internal Medicine with a focus on gastroenterology, Berlin, Germany

SKILLS

Written and verbal communication
Native language German, fluent English and French, beginners Turkish.
Test of English as a Foreign Language (TOEFL Test) in Mar '09, result 113 out of 120.

XI Acknowledgements

The experimental part was conducted between September 2007 and July 2008 in the Clinical Research Unit of the Department for Gynecology and Obstetrics of the Technical University of Munich, Germany under the supervision of Prof. Dr. Viktor Magdolen.

First of all I want to thank Prof. Dr. Viktor Magdolen for providing me with the topic of the thesis, for answering any question that arose and for giving me new directions and ideas that helped the progress of my work.

Furthermore, I thank Prof. Dr. Dr. Manfred Schmitt, who is the head of the Clinical Research Group, for his help with this work.

I would like to give special thanks to Sandra Baur, who was always very supportive and who taught me new technical methods. She was there to answer my questions, especially during protein purification and refolding, as well as PAR-experiments. In addition I also want to say thank you to Sabine Creutzburg, whom I admire very much for her laboratory skills and her patience.

Sincere thanks also to Dr. Mekdes Debela, who helped me with my experiments and who introduced me to a number of technical methods.

I also want to thank Dr. Martina Müller for her company and good ideas during talks and discussions at laboratory meetings.

Thanks to the lab of Dr. Josef Kellermann and Prof. Dr. Friedrich Lottspeich at the Max Planck Institute for Biochemistry, Martinsried, Germany (Section Proteinanalysis), who performed the N-terminal sequencing.

Finally, I want to thank my family and friends, who have always been very supportive.