HUMAN PROSTATE-SPECIFIC ANTIGEN AND GLANDULAR KALLIKREIN 2

Production and characterization of the recombinant proteins, and association with prostate cancer

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OULU 2002
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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 9 of the University Hospital of Oulu, on September 6th, 2002, at 12 noon.
Herrala, Annakaisa, Human prostate-specific antigen and glandular kallikrein 2
Production and characterization of the recombinant proteins, and association with prostate cancer
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Abstract

Human prostate-specific antigen (hPSA, KLK3) and glandular kallikrein 2 (hK2, KLK2), two members of a large human tissue kallikrein enzyme family, were produced as recombinant mature proteins for the first time and characterized. Furthermore, their association with prostate cancer was studied. Both proteins were produced with baculovirus expression vector system in pilot-scale using bioreactors. Recombinant hPSA was either active with chymotrypsin-like activity or inactive with incorrect processing of N-terminus. The molecular weight of active recombinant hPSA was 31 kD and it formed stable complexes with serine protease inhibitors, α1-antichymotrypsin (ACT) and α2-macroglobulin (α2M). Two polymorphic forms of KLK2, Arg226hK2 and Trp226hK2, were found. The recombinant Arg226hK2 had trypsin-like activity, while recombinant Trp226hK2 was inactive. The Arg226hK2 was labile with low production yields. The molecular weights of hK2 polymorphic forms were 33 kD.

hPSA isoforms secreted by prostate cancer cells, LNCaP, were isolated and characterized. These proteins were N-terminally heterogeneous: 10-60% of LNCaP-PSAs were correctly processed. Molecular modeling suggested that the additions or deletions of two or four N-terminal amino acids could affect the three-dimensional structure and reduce the activity of LNCaP-PSA. Active isoforms had chymotrypsin-like activity and formed stable complexes with ACT and α2M.

The expression of hPSA and hK2 was studied with in situ hybridization and immunohistochemistry techniques in benign and cancerous prostate tissue. hK2 mRNA was expressed at a significantly higher level in prostate cancer tissue than in benign prostate tissue (P < 0.0005). The hPSA mRNA expression levels were reversed (P = 0.06). In benign tissue, the mean level of hK2 mRNA was 82% of the respective value of hPSA (P < 0.003), whereas in tumor tissue the mean hK2 expression level was 21% higher than that of hPSA (P < 0.01). The results at protein level supported the mRNA findings. There was a correlation between hPSA and hK2 mRNA levels in both benign (r = 0.735; P < 0.01) and malignant (r = 0.767; P < 0.01) prostate tissue. It was shown with competitively differential PCR that the KLK2 gene was amplified in prostate tumor tissue, while the KLK3 gene was not. These results suggest that hK2 and hPSA have a diverse value in the diagnosis of prostate cancer.

Keywords: prostatic neoplasms, prostate-specific antigen, prostate-specific glandular kallikrein 2, recombinant proteins, tissue kallikreins, prostatic hyperplasia, gene amplification
To Ville-Waltteri, Arttu, Onni & Siiri
Acknowledgements

The present study was carried out at Biocenter Oulu and at the Research Center for Molecular Endocrinology, which also acts as a WHO Collaborating Centre for Research on Reproductive Health, Faculty of Medicine, University of Oulu.

I wish to express my sincere gratitude to my supervisor, Professor Pirkko Vihko for giving me the opportunity to prepare my thesis in her research group with excellent facilities for research work, and for her guidance and support during these years. I also want to thank the President of the Academy of Finland, Professor Reijo Vihko, for his support during the early stages of this work.

I am grateful to Professor Jorma Isola and Docent Antti Pajunen for their constructive criticism of this thesis. I also wish to thank Anna Vuolteenaho for the careful language revision of this thesis manuscript.

I am deeply grateful for the additional supervision provided by Riitta Kurkela and Katja Porvar. Their support, expert knowledge, co-authoring in this work, patience and especially the friendship of the dear "Kollegas" will always stay within my heart.

I am also indebted to all my other co-authors Pirkko Henttu, Heini Nal, Ritva Isomäki, Professor Mauno Vihinen, Docent Nisse Kalkkinen and Atte Kyllönen for their valuable contribution to this work.

I am grateful for the expert technical assistance of Pirkko Ruokojärvi and Marja-Liisa Norrena in cell culture and for Marja-Riitta Hurnasti, Helmi Konola and Mirja Mäkeläinen in protein and molecular biology laboratories.

I owe my gratitude also to Saini Sydänmäa for her friendship and guidance to real laboratory work when I joined the "Prostate girls". All the members of "Prostate girls and boys" are remembered with warm feelings. I want to express my thanks to Helena Kaija, Kari Juntunen, Anitta Pulkka and Svea Törn, the members of the former "Protein lab", for their support and friendship. I wish to extend my sincere thanks to all my co-workers, Finnish and foreign, past and present, at the Research Center for Molecular Endocrinology and "Vihkos' groups" for sharing their expertise, friendship and knowledge.

I also wish to thank Anja Raatikainen and Sirpa Annanperä for their administrative help, Juhani Heikkilä and Jaakko Heikkilä for their help and patience with the computer.
problems. All the personnel of the Medical Library and the Photography Laboratory of Medical Faculty are acknowledged for their fast and pleasant services.

Professor Marta Menjivar, Bertha Rodriguez, Yan Li, Professor Sergio Ghersevich, and Professor Yun-shang Piao, thank you for your friendship, it is good to know that I have family all over the world.

Riitta, the trips outside of the laboratory have been a way to relax and gain new unforgettable experiences. I will always remember our trip to Norway, "Mr. Murphy" tried to rule throughout the whole trip, "but WHO cares…"

I want to express my thanks to my late mother for her encouragement and love and to my brother Erkki and his wife, Minna and their children Ville-Walterti, Arttu, Onni and Siiri; the "active holidays" with you have been a perfect escape from the research world. The friends outside of the laboratory are acknowledged, too.

This study was mainly supported by Biocenter Oulu and the Research Council of Finnish Academy, The Technology Development Centre of Finland (TEKES), the Cancer Foundation of Northern Finland, the Ida Montin Foundation, and Research and Science Foundation of Farmos.

Oulu, June 2002

Annakaisa Herrala
Abbreviations

α2M  α2-macroglobulin
AcNPV  *Autographa californica* nuclear polyhedrosis virus
ACT  α1-antichymotrypsin
BEV  baculovirus expression vector
BPH  benign prostatic hyperplasia
cDNA  complementary deoxyribonucleic acid
CD-PCR  competitively differential polymerase chain reaction
CNS  central nervous system
EMSP1  enamel matrix serine protease 1
EST  expressed sequence tag
FISH  fluorescence *in situ* hybridization
FPLC  fast protein liquid chromatography
h  human
hGK-1  human glandular kallikrein-1
HGNC  HUGO Gene Nomenclature Committee
HSCCE  human stratum corneum chymotryptic enzyme
HSCTE  human stratum corneum tryptic enzyme
HUGO  The Human Genome Organization
K2  prostate-specific glandular kallikrein
KLK1  gene encoding tissue/renal kallikrein
KLK2  gene encoding prostate-specific glandular kallikrein
KLK3  gene encoding prostate-specific antigen
KLK-L  kallikrein-like
IEF  isoelectric focusing
kb  kilobases
kDa  kilodalton
NES1  normal epithelial cell-specific 1 gene
nt  nucleotide
PAGE  polyacrylamide gel electrophoresis
PAP  prostatic acid phosphatase
PCR  polymerase chain reaction
PSA    prostate-specific antigen
PSMA   prostate-specific membrane antigen
PRSS   protease serine
PRSSL  PRSS-like
RT-PCR reverse transcriptase polymerase chain reaction
SCCE   stratum corneum chymotryptic enzyme
SDS    sodium dodecyl sulfate
Sf     Spodoptera frugiperda
TADG-14 tumor-associated differentially expressed gene-14
TLSP   trypsin-like serine protease
UPA    urokinase-type plasminogen activator
List of original publications

This thesis is based on following articles, which are referred to in the text by their Roman numerals:


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Original publications
1 Introduction

The human tissue or glandular kallikrein gene family was thought to be formed of only three members, the classical kallikreins, tissue/renal kallikrein (KLK1), prostate-specific antigen (hKLK3, PSA) and human prostate-specific glandular kallikrein 2 (hKLK2, hK2) (Riegman et al. 1992). During the last few years several new members of the human kallikrein gene family have been discovered and now the size of the gene family is 15, including new kallikreins KLK4-KLK15. All the human kallikrein genes are located at the same chromosomal locus, 19q13.4, within 320 kb region of the chromosome (Yousef et al. 2000a, 2001a, Yousef & Diamandis 2001, Clements et al. 2001) (Figure 1). Human kallikreins share many common features (Yousef & Diamandis 2001). There is significant homology on nucleotide and amino acid levels (40-80%) and similar structural and genetical organization. All kallikrein genes have five similar or equal sized coding exons, with the exception of some kallikreins with one or more untranslated 5'-exons. The intron phases are fully conserved. There is a conserved catalytic triad of histidine, aspartatic acid and serine or asparagine present in all kallikreins. They encode putative serine proteases with trypsin-like or chymotrypsin-like activity. Many of the kallikreins are under steroid hormone regulation. Some of these kallikreins are expressed differently in benign tissue versus malignant tissue, which make them potential markers for diseases.

Prostate cancer is the most frequently diagnosed malignancy among men. 25% of all cancers diagnosed in Finnish men are of prostate origin (Finnish Cancer Registry 2000). Human PSA is a mainstay in the diagnosis and follow-up of prostate cancer, and hK2 is a new potential candidate to improve the diagnosis of the disease. The homology between hPSA and hK2 is about 78% (Lundwall & Lilja 1987, Henttu & Vihko 1989, 1994). Regardless of the name, neither hPSA nor hK2 are expressed solely in prostate, but also in various other tissues, such as breast.

The development of gene technologies has lead to a situation where recombinant proteins are involved in our lives as new effective medicines, modified food or diagnostic supplies. Recombinant protein production in bacteria, yeast, plants, animal and mammalian cells has made it possible to access substances, the purification of which from human or animal tissues for commercial purposes is either difficult or unethical. The suitability of recombinant proteins as standards or as antigens in antibody generation for diagnostic assay development is currently under increasing use.
The present work focuses on two members of the tissue kallikrein family hPSA and hK2. Both enzymes have been produced as recombinant proteins and were compared to natural hPSA from seminal fluid, while natural hK2 was not available. The high homology between hPSA and hK2 proteins has made it difficult to purify them as individual proteins from natural sources. hK2 contamination of hPSA purified from seminal fluid has been shown (Frenette et al. 1998a, Wang et al. 1999) and it has been difficult to obtain antibodies specific for the individual serine proteases (II). Due to these facts, the recombinant forms of hPSA and hK2 allow the characterization of their specific properties. The knowledge concerning the specific characteristics of hPSA and hK2 and the generation of specific monoclonal antibodies help to evaluate their association and significance in the pathological states of the prostate.
2 Review of the literature

2.1 Serine proteases

Proteases or proteolytic enzymes are essential in organisms, from bacteria and viruses to mammals. Proteases digest and degrade proteins by hydrolyzing peptide bonds, and they also perform special processes in complicated organisms. Proteolytic enzymes are synthesized as zymogens, larger inactive precursors; this way the unwanted protein degradation is restrained (Khan & James and the references therein 1998). During evolution formed serine proteases (EC. 3.4.21) two main families, trypsin-like serine proteases and subtilisin-like pro-protein convertases (Creighton 1993). Duplication of the ancestral gene has been considered the reason for the arising of trypsin-like serine protease genes through mutations to form related proteases and new sub-families, which are divided into serine, cysteine (thiol), aspartatic (carboxyl), metalloproteases. The classification is based on the functional group in their active sites (Barrett 1980). Serine proteases have a common catalytic triad consisting of three amino acids: serine (nucleophile), aspartate (electrophile), and histidine (base) in their active site (Neurath 1984, Young et al. 1978). Based on their intron positions serine proteases are classified into five groups: 1) No introns interrupt the exon coding for the catalytic triad, haptoglobin gene, 2) each gene contains an intron just downstream from the codon for histidine residue at active site, a second intron downstream from the exon containing the aspartic acid residue of active site and a third intron just upstream from the exon containing serine of the active site, e.g. trypsinogen, chymotrypsinogen, kallikrein and proelastase, 3) the gene containing seven introns interrupting the exons coding the catalytic region, complement factor B gene, 4) two introns resulting in a large exon that contains both the active site aspartatic acid and serine residues, factor X, factor IX and protein C genes, and 5) the prothrombin gene, which is different from all other serine protease genes mentioned above (Irwin et al.1988). There is aspartate in the binding pocket of trypsin-like serine proteases, forming a strong electrostatic bond with arginine or lysine amino acid residues, usually located at the carboxyl-terminal part of the cleavage site (Yousef & Diamandis and the references therein, 2001).
2.2 Kallikrein gene families

The glandular or tissue kallikreins are a sub-family of serine proteases, with a high degree of substrate specificity and diverse expression in various tissues and biological fluids. The term "kallikrein" appeared in the literature for the first time in the 1930s, when large amounts of protease enzymes were found in pancreas isolates (pancreas is "Kallikreas" in Greek) (Kraut et al. 1930, Werle 1934). Nowadays kallikrein enzymes are divided into two groups, plasma (EC 3.4.21.34) and tissue (EC 3.4.21.35) kallikreins, which differ significantly in their molecular weight, substrate specificity, immunological characteristics, gene structure, and type of the kinin released. Plasma kallikrein is expressed solely in liver and it is involved in blood clotting, fibrinolysis, regulation of blood pressure and inflammatory reactions (Bhoola et al. 1992). Tissue kallikreins are a large group of enzymes which have substantial similarities at gene and protein level. Tissue kallikreins are involved in the post-translational processing of the polypeptides (like kininogen) and releasing potential biologically active peptides (like kinin) (Clements 1989, 1997). Tissue kallikreins are also called kininogenases. Kininogenases or kininases are enzymes that inactivate kinins (Yousef & Diamandis and the references therein, 2001). Pancreatic/renal or K1 (new nomenclature) kallikrein enzyme is the only one among human and animal tissue kallikreins possessing kininogenase activity, releasing lysyl-bradykinin from kininogen. hK1 has also an effect on blood pressure, electrolyte balance and inflammation, and it may also digest other substrates, like growth factors, hormones, and cytokines (Bhoola et al. 1992). The kallikrein-kinin system has been shown to activate angiogenesis in an in vivo mouse model (Emanueli et al. 2001). The concept "tissue kallikrein" does not only refer the enzymes with the above-mentioned functions, but also to group of enzymes with conserved gene and protein structures that are also located at same gene locus as the KLK1 gene.

The size of the tissue kallikrein gene family varies between species. The genes are located as clusters in the same chromosomal locus. In the house mouse, the kallikrein genes are clustered at chromosome 7 in a single locus (Mason et al. 1983, Evans et al. 1987). The mouse kallikrein gene family has at least 24 members, 14 of which seem to be produced as functional proteins, while the rest are pseudogenes (Mason et al. 1983, van Leeuwen et al. 1986, Evans et al. 1987, Drinkwater et al. 1987). In the rat genome there is also a large kallikrein gene family, 15-20 genes, and at least 10-11 of the genes produce functional proteins, which are expressed with different patterns in various organs (Wines et al. 1989, MacDonald et al. 1996). The guinea pig tissue kallikrein family is smaller than in other rodents, it has only three members (Fiedler et al. 1999).
2.2.1 Human kallikreins

2.2.1.1 Characteristics of human kallikreins

The human tissue kallikrein gene family was thought to be formed of three genes (Riegman et al. 1992), but during the last few years studies on new kallikrein-like genes have shown that there are at least 15 members in this family. It is believed that members of this family are developed through gene or chromosomal duplication from a common ancestor gene, while they have similar activities or predicted activities (all of them have not yet been isolated in their native form or produced as recombinant protein), have conversed primary and tertiary structures (Gan et al. 2000). Tissue kallikrein genes are clustered on chromosome 19 (Riegman et al. 1992, Gan et al. 2000, Harvey et al. 2000, Yousef et al. 2000a), like other gene clusters, like the granzyme gene cluster on chromosome 14 (Barros et al. 1996) and the trypsinogen loci on chromosomes 7 and 9 (Rowen et al. 1996). All tissue kallikrein genes are located within the 320 kb region (Gan et al. 2000, Harvey et al. 2000, Yousef et al. 1999a, 2000a). Kallikreins are heat-stable glycoproteins with a single polypeptide chain, with molecular weight varying between 27 to 40 kDa (Clements 1989 and the references therein). Kallikreins are serine proteases like elastase, thrombin, plasmin, chymotrypsin and trypsin, with the characteristic serine residue and conserved Gly-Asp-Ser-Gly amino acid sequence in their catalytic site (Neurath et al. 1967). There are 29 conserved or invariant amino acids surrounding the catalytic site, which are also present in tissue kallikreins (Dayhoff 1976).

Three of the known human kallikreins are called classical kallikreins because of their earlier discovery, and the 12 kallikrein genes discovered during the last few years are termed new kallikreins. Yousef with co-workers (2000a) showed, with the linear genomic sequences around chromosome 19q13.3-q13.4 generated by the Lawrence Livermore National Laboratory, the precise location of three classical and 12 new kallikrein genes. The chromosomal organization of human kallikrein genes is presented in Figure 1. The nomenclature for kallikrein genes, especially for new genes has been miscellaneous. Some of the new kallikreins were nominated according to The Human Genome Organization's (HUGO) proposed guidelines for the nomenclature of serine proteases with PRSS, and some with KLK prefixes. An international group of scientists suggested uniform nomenclature for all the human tissue kallikreins (Diamandis et al. 2000a), which generated vivid discussion on the HUGO Gene Nomenclature Committee home pages on the Internet (HGNC 2000), especially in the case of hKLK4 (Table 1).

All human tissue kallikreins share common features. All tissue kallikrein genes are located at 19q13.4 chromosomal region, they encode putative serine proteases, with a catalytic triad of amino acids in their active site. Histidine, aspartic acid, and serine are in conserved positions for trypsin-like activity, or asparagine instead of serine, predicting chymotrypsin-like activity. All genes have five coding exons, with identical or similar sizes (some have one or more untranslated exons). Intron phases are conserved for all tissue kallikrein genes. There are significant, 40-80%, sequence homologies at DNA and protein levels for all kallikreins, but within the new kallikrein genes the homologies are
<table>
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<th>Gene symbol</th>
<th>Previous gene symbol</th>
<th>Protein symbol</th>
<th>Other protein symbols/names*</th>
<th>Accession number at the GenBank</th>
<th>Reference</th>
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<td>KLK1</td>
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The table is modified from Yousef & Diamandis (2001) and HGNC gene nomenclature homepage (2000).

* Full names are presented in abbreviations.
less than 25-44% (Clements et al. 2001). The major difference between classical and new kallikreins is that there are extra exons (either 5' or 3') present in many of the new kallikreins (Luo et al. 1998, Hu et al. 2000, Mitsui et al. 2000, Yousef & Diamandis 2001, Yousef et al. 1999c, 2000c, d).

2.2.1.2 Classical kallikreins

There are three so-called classical kallikreins: tissue/renal/pancreatic kallikrein, prostate-specific glandular kallikrein and prostate-specific antigen. For prostate-specific antigen the most known symbol hPSA will be used throughout this review. hK1 is secreted from the kidney, pancreas and salivary glands (Schachter 1979). hK1 is the only member of kallikrein family which is involved in the regulation of blood flow, sodium equilibrium, inflammation, and cell proliferation (Bhoola et al. 1992). Nucleotide sequences for classical kallikreins were clarified in the 1980s, KLK1 (Baker & Shine 1985, Fukushima et al. 1985), KLK2 (Schedlich et al. 1987) and KLK3 (Wang et al. 1981). These three kallikreins have high homologies between each other in their DNA and protein levels; KLK1 and KLK3 have about 60%, KLK1 and KLK2 66%, while KLK2 and KLK3 have about 80% homology (Henttu & Vihko 1989). The genes cluster in a 60 kb region on human chromosome 19q13.3-q13.4 (Yousef et al. 2000a) as seen in Figure 1. The "Kallikrein loop", a 9-11 amino acid peptide sequence, is only found in classical kallikreins. This loop precedes the aspartatic acid in the active site/catalytic triad (Yousef & Diamandis 2001). Classical kallikreins have also splice variant forms. For KLK1 gene a new transcript has been isolated from colon (Chen et al. 1994). The KLK2 gene has also splice variants (Riegman et al. 1991, Liu et al. 1999). KLK3 has been described to have splice variants in addition to the main 1.6-kb transcript (Riegman et al. 1989, Henttu et al. 1990, Heuzé et al. 1999).

2.2.1.3 New kallikreins

The human kallikrein gene family was thought to be composed of 3-4 (Fukushima et al. 1985, Baker & Shine 1985) up to 19 genes (Murray et al. 1990). New technologies and information available (Human Genome Project) has made it possible to identify or predict the existence of new genes, and during the past few years new members of the human tissue kallikrein family have been discovered. The genes in the family, including the classical kallikreins, are named KLK1-KLK15 and the proteins they encode are termed hK1-hK15 (Table 1). KLK4-KLK15 genes are less related (25-44%) and do not have the usual kallikrein loop of classical kallikreins (Clements et al. 2001). The expression of new kallikreins in different tissues has been studied mainly using RT-PCR techniques (Gan et al. 2000 and the references therein, Clements et al. 2001 and the references therein) and a summary of this data is presented Figure 2.
KLK4 with protein symbol hK4 was independently cloned by two groups (Nelson et al. 1999, Stephenson et al. 1999). It is a significantly divergent member of the human tissue kallikrein family. KLK4 is located down-stream from KLK2 gene transcribing
Fig. 1. The localization of human tissue kallikrein gene family at chromosomal locus 19q13.3-13.4. The kallikrein genes are represented as boxes bearing the names of the respective genes. The length of the gene (in base pairs, bp) is marked on the right-hand side of the respective box. The distances (in bp) between genes are shown between "boxes". Arrows on the left of each gene point to the direction of transcription. Modified from Yousef & Diamandis (2000) and Clements et al. (2001).
the same direction as KLK1 (Stephenson et al. 1999). The exon necessary for signal peptide formation is not present, and the green fluorescent tagged KLK4 is localized perinuclearly, suggesting that KLK4 has intracellular functions contrary to other members of the kallikrein gene family (Korkmaz et al. 2001).

KLK5 was identified from stratum corneum extracts (Brattsand & Egelrud 1999) and with positional candidate approach it was shown to be a member of the KLK family (Yousef & Diamandis 1999). The hK5 enzyme is proposed to be a part of turnover of stratum corneum and desquamation (Ekholm et al. 2000).

The KLK6 gene was discovered by several research groups (Anisowicz et al. 1996, Little et al. 1997, Yamashiro et al. 1997). KLK6 gene has high homology to trypsin (Anisowicz et al. 1996) and it might also possess amyloidogenic activity (Little et al. 1997). KLK6 has seven exons, two first ones are untranslated (Yousef et al. 1999c).

KLK7 was first linked to the desquamation of stratum corneum (Lundstrom & Egelrud, 1991). It was later cloned and characterized by two groups (Hansson et al. 1994, Yousef et al. 2000b) to be part of the tissue kallikrein gene family with chymotryptsin-like instead of trypsin-like activity, because aspartatic acid is replaced with asparagine in substrate binding pocket (Ekholm & Egelrud 1999).

KLK8 was first thought only to be a brain-related enzyme (Yoshida et al. 1998a), but it was found to be expressed in ovarian carcinoma (Underwood et al. 1999). KLK8 has two splice variants (Mitsui et al. 1999, Magklara et al. 2001), which are expressed abundantly in several normal human tissues, but mainly in human brain. KLK8 cDNA and the predicted amino acid sequence have 72% identity to corresponding one in mouse (Yoshida et al. 1998a).

When the human kallikrein gene locus was characterized, the KLK9 gene was discovered. The predicted enzyme activity for hK9 enzyme is chymotryptsin-like, while serine is replaced by aspartate in the catalytic triad (Yousef & Diamandis 2000).

KLK10 was isolated with subtractive hybridization between a nontumorigenic and tumorigenic breast cancer cell line (Liu et al. 1996). KLK10 acts as tumor suppressor in prostate, breast and possible other epithelial cells (Goyal et al. 1998). KLK10 has six exons, one untranslated (Luo et al. 1998). KLK10 gene expression is up-regulated by estrogens, androgens and progestins when studied with BT-474 breast cancer cell line (Luo et al. 2000). An assay measuring the free fraction of K10 protein in biological fluids and tissues has been developed using recombinant K10 (Luo et al. 2001a).

KLK11 was first cloned from human hippocampal cDNA with PCR (Yoshida et al. 1998b), and it is expressed in human skin, salivary gland, stomach, prostate, intestine and brain (Yousef et al. 2000c). KLK11 has six exons (first one untranslated) and five identical introns to other kallikreins (Yousef et al. 2000c). KLK11 has two splice forms, with a difference in their N-terminal part, which are expressed primarily in either the brain or prostate. Serine in catalytic triad predicts trypsin-like activity for hK11 (Mitsui et al. 2000).

The sequence alignment test indicated that KLK12 has seven exons, at least one of them untranslated. One extra 3' exon was also found, which has not been reported for any other members of kallikrein family. KLK12 has three splice variant forms. Using cDNA from 26 different tissues as template, three distinct bands were observed in many tissues.
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Fig. 2. Expression of kallikreins in different tissues. The expression detected is marked with a grey square, high expression detected is marked with a black square and no expression detected is marked with a white square. For more information see text.
The first is "classical form" with the typical five exons and four introns. The second is KLK12-related protein-1, in which the last exon is split into two separate exons with an additional intron. KLK12-related protein-1 is a protein 254 amino acids long, six amino acids longer than classical KLK12. The third is KLK12-related protein-2, which is otherwise similar to classical KLK12, but the fourth exon is missing (Yousef et al. 2000d).

The KLK13 gene was identified with the positional candidate gene approach. It has the typical gene structure for kallikreins with five coding exons. This gene is mainly expressed in prostate, salivary gland, breast, and testis. Aspartate in position 239 predicts that KLK13 protein is likely to have trypsin-like activity (Yousef et al. 2000e). KLK13 has also five splice variants, which are differentially expressed in testis and testicular cancer (Chan et al. 2001).

KLK14 has seven exons, five of which are coding exons (Hooper et al. 2001, Yousef et al. 2001b). KLK14 is expressed in various tissues, but mainly in central nervous system (CNS), which indicates that KLK14 might be involved in brain physiology. Aspartate at position 198 predicts trypsin-like activity (Yousef et al. 2001b).

KLK15 was found to be located between KLK1 and KLK3 at chromosome 19q13.4. There are five coding exons in KLK15. The possible presence of upstream-untranslated exon(s) could not be ruled out (Yousef et al. 2001c). Glutamic acid at the position 203 suggests that hK15 has unique substrate specificity. KLK15 has evenly distributed hydrophobic regions in the polypeptide chain like globular proteins, similarly to other serine proteases. At positions 148-155 hK15 has a unique 8-amino acid loop (HNEPGTAG). Out of the 29 conserved amino acids surrounding the active site, 28 were found in KLK15. One of the non-conserved amino acids (serine 173 instead of proline) is also found in hK5 and hK12 proteins, representing a conserved evolutionary change to a protein of the same group, according to protein evolution studies. The KLK15 gene is expressed in various tissues, with highest levels in the thyroid gland, and showing high expression in high-grade prostate tumors. The KLK15 gene might have a function in the physiology of normal thyroid gland since the expression of the gene is so high. KLK15 gene also has two splice variants which probably express truncated proteins. With phylogenetic studies KLK15 was grouped with KLK9 and KLK11 (Yousef et al. 2001c).

2.2.1.4 Kallikreins in diseases/malignancies

The exact role and involvement of tissue kallikreins in human diseases is not clear. Serine proteases are involved in tumor progression, such as invasion, proliferation and tumor metastasis (Tryggvason et al. 1987, DeClerck et al. 1997, Carroll & Binder 1999, Wolf et al. 2001), but there are data supporting contrary functions of serine proteases in diseases. The serine proteases act as tumor suppressors (Goyal et al. 1998), as antiangiogenic factors (Fortier et al. 1999), as apoptotic molecules (Balbay et al. 1999) or negatively regulate cell growth (Lai et al. 1996).

hK1 as a part of the kallikrein kinin system is involved in hypertension (Margolius et al. 1974), inflammation (Clements 1997), pancreatitis (Griesbacher & Lembeck 1997),
renal diseases (Horwitz et al. 1978, Holland et al. 1980) and different types of cancers (Berg et al. 1985, Jones et al. 1989, 1990). High expression of the KLK4 gene has been reported in ovarian tumors, especially in aggressive forms of ovarian carcinomas (Dong et al. 2001, Obiezu et al. 2001). The expression of KLK4 gene is high in prostate tissue (Nelson et al. 1999, Yousef et al. 1999b, Harvey et al. 2000), but the connection between hKLK4 and prostate cancer has not been shown yet. KLK5 (Kim et al. 2001), KLK6 (Anisowicz et al. 1996, Diamandis et al. 2000b), KLK7 (Tanimoto et al. 1999), KLK8 (Underwood et al. 1999, Magklara et al. 2001) and KLK10 (Luo et al. 2001b,c) are also expressed in high levels in ovarian cancer and could be future ovarian cancer markers. KLK6 is expressed in primary mammary carcinoma cell lines (Anisowicz et al. 1996) and the connection of KLK6 to Alzheimer's disease has been shown with its amyloidogenic activity (Little et al. 1997, Diamandis et al. 2000b). KLK7 was first linked to the desquamation of stratum corneum (Lundstrom & Egelrud, 1991), and it is suggested that it has a role in skin diseases, like pathological keratinization and psoriasis (Sondell et al. 1996, Ekholm & Egelrud 1999). KLK8 has also high expression in Alzheimer's disease (Shimizu-Okabe et al. 2001) and its involvement in diseases of the CNS has been reported (Akita et al. 1997, Scarisbrick et al. 1997, Momota et al. 1998, Kishi et al. 1999, Mitsui et al. 1999, Yoshida & Shiosaka 1999). KLK9 is expressed at significantly higher levels in early stages of ovarian carcinoma (Yousef et al. 2001d). KLK10 expression is down-regulated in aggressive forms of prostate cancer (Luo & Diamandis 2000). It has been shown in animal models that KLK10 acts as a tumor suppressor in breast cancer (Liu et al. 1996, Goyal et al. 1998). hK10 has been suggested to be a marker for ovarian cancer (Luo et al. 2001b, c). Elevated serum levels of hK11 have been detected in ovarian and prostate cancer (Diamandis et al. 2002). KLK12-14 expression is low in breast cancer (Yousef et al. 2000b, Yousef et al. 2000d, e, 2001b). KLK15 shows higher expression in more aggressive, higher stage prostate cancers (Yousef et al. 2001c).

The role and suitability of new kallikreins as a marker for diseases, particularly in cancers, will be under investigations for next the few years. The role of hK2 and hPSA in diseases is discussed later in this review.

2.2.1.5 The steroid hormone regulation of kallikreins

identified and their function has been studied (Luke & Coffey 1994, Schuur et al. 1996, Cleutjens et al. 1997).

The KLK4 gene is up-regulated by androgens in LNCaP prostate cancer cells and by androgens and progestins in BT-474 breast cancer cells (Nelson et al. 1999, Yousef et al. 1999b). Putative androgen response elements have been identified from the proximal promoter area of KLK4 (Stephenson et al. 1999). KLK5 expression is up-regulated with estrogens and progestins in breast cancer cell lines (Yousef & Diamandis 1999). Estrogens and progestins and to a lesser extent androgens up-regulate KLK6 expression in breast cancer cells (Yousef et al. 1999c). KLK7 expression is up-regulated in breast cancer cells by estrogens and glucocorticoids (Yousef et al. 2000b). KLK9 expression is up-regulated in breast cancer cell line by progestins, estrogens and androgens, the highest up-regulation having been detected with progestin induction (Yousef & Diamandis 2000).

KLK10 expression is up-regulated in breast cancer cell line BT-474 by steroid hormone stimulation with following potency: estrogens > androgens > progestins (Luo et al. 2000). The expression of KLK11 gene was shown to be regulated with steroid hormones in the same cells (Yousef et al. 2000c). KLK12 gene expression is up-regulated by androgens and progestins in LNCaP prostate cancer cells, whereas in two breast cancer cell lines KLK12 expression is up-regulated by steroid hormones with the following potencies: estrogens > androgens > progestins in BT-474 cells and androgens > progestins > estrogens in T-47D cells (Yousef et al. 2000d). KLK13 gene expression is up-regulated by androgens and progestins and to a lesser extent by estrogens in BT-474 cells (Yousef et al. 2000e). KLK15 gene expression is up-regulated in LNCaP cells; the highest induction was obtained with dihydrotestosterone and lowest with aldosterone (Yousef et al. 2001c).

2.3 Human prostate-specific antigen

2.3.1 hPSA expression and tissue distribution

In 1971, Hara et al. described for the first time γ-semioprotein, later termed, prostate-specific antigen, hPSA or hK3, and this enzyme protein was purified by Sensabaugh in 1978 from semen in order to utilize it as a forensic marker in rape cases. An identical protein was confirmed to be hPSA (Papsidero et al. 1980), was isolated from serum of prostate cancer patients. hPSA is expressed as 261 amino acid preproprotein, with a 17 amino acid signal sequence and a 7 amino acid activation peptide (Lundwall & Lilja 1987, Riegman et al. 1988, Henttu & Vihko 1989). The molecular weight of the free hPSA molecule is 33 or 34 kDa (depending on source of information) in human seminal plasma and serum (Wang et al. 1979, 1983, Schaller et al. 1987, Zhang et al. 1995, III). hPSA is a glycoprotein with one carbohydrate side chain (Schaller et al. 1987). hPSA is mainly expressed in epithelial cells of the lumen in the prostate gland (Wang et al. 1981, Sinha et al. 1987, Hakalahti et al. 1993). The name prostate-specific antigen is actually misleading. hPSA has been found to be expressed in many non-prostatic normal or tumor tissues, like breast or breast cancer tissue and breast cancer cells, colon, kidney, liver,

Breast tissue (Yu et al. 1996, Diamandis & Yu 1997, Parish 1998) and placenta during pregnancy (Mannello et al. 1998, Malatesta et al. 2000) have also been to be suggested the main source of female hPSA. hPSA contents have been measured from other body fluids besides serum or seminal fluid, like colostrum and milk (Yu & Diamandis 1994, Lövgren et al. 1999a), breast cyst fluid (Diamandis et al. 1996), amniotic fluid (Yu & Diamandis 1995, Melegos et al. 1996) and urine (Pannek et al. 1997, Obiezu et al. 2000).

The normal prostate contains hPSA levels about million-fold higher than serum (Rittenhouse et al. 1998). The normal serum levels of hPSA in males are under 4 µg/L (Vihko et al. 1990, Oesterling 1991), while the hPSA levels in the seminal fluid are 10^6-fold higher, 0.2-5 mg/ml (Sensabaugh 1978, Wang et al. 1979, 1981). The hPSA concentration in female serum is much lower than in male, ~0.2-0.3 µg/L (Vihko et al. 1990, Malatesta et al. 2000).

### 2.3.2 The function of hPSA

Structural studies showed that at substrate binding pocket aspartate 189 is replaced with serine giving chymotrypsin-like activity for hPSA (Villoutreix et al. 1994). The whole concept of the physiological function of hPSA is not very clear in the entire human body. The first known physiological function of hPSA was the liquefying of seminal clot after ejaculation by digestion of seminogelins and fibronectin and enhancing sperm mobility (Lilja 1987). Zinc ion has been reported to regulate hPSA activity by binding tightly to the enzyme and inactivating it in vitro; also Cu^2+, Hg^2+, Co^2+ and Cd^2+ had an inhibitory effect on hPSA (Malm et al. 2000). In seminal fluid hPSA has also been reported to digest a glycoprotein and release a kinin-like substance that stimulates smooth muscle contraction (Fichtner et al. 1996). hPSA has been studied in fetal development, it is produced at higher concentrations during gestation weeks 14-20 by the fetus (Melegos et al. 1996). In vitro studies suggest that hPSA takes part in the regulation of prostatic fibromuscular growth through modifying interactions between insulin-like growth factor and insulin-like growth factor-binding protein (Sutkowski et al. 1999). It has been demonstrated that hPSA changes the biological activity of parathyroid hormone-related protein (PTHrP) by cleaving its domains. This proteolytic processing of PTHrP may be important for the regulation of prostate growth and differentiation (Iwamura et al. 1996), and it also suggests a role for PTHrP in the metastasis of prostate cancer to bone (Cramer et al. 1996). Diamandis (2000 and references therein) has in his recent review speculated a role for hPSA in cancer, asking whether if hPSA is a cancer fighter and important indicator of the stage of cancer, or a promoter of tumor progression. The suggestions for
other physiological functions of hPSA vary between the hypothesis that hPSA has cancer protective or inhibitory effects, such as possessing anti-angiogenic activity (Pretlow et al. 1991, Heidtmann et al. 1999, Fortier et al. 1999). On the other hand, it has been proposed that hPSA is a cancer promoting factor, having chymotrypsin-like activity and making formation of prostate cancer metastasis easier (Webber et al. 1995). Since it has mitogenic activity and stimulates cell detachment, hPSA is proposed to promote and be involved in prostate tumor growth and its spreading into bones (Killian et al. 1993). hPSA expression has been shown to be restricted to primates (Rittenhouse et al. 1998) and because of that mouse knock-out models are not conceivable for studying functions of PSA.

### 2.3.3 hPSA in prostatic diseases

The value of hPSA in the diagnosis and follow-up of prostate cancer was noticed soon after its discovery in 1970. The Food and Drug Administration (FDA) has accepted hPSA as a first tumor marker for diagnosing prostate cancer in population screening programs (Nightingale 1994). The most important use of hPSA is the post therapy/treatment follow-up. hPSA concentrations increase in the serum with the patient's age and prostate volume (Vihko et al. 1990).

The basis of using hPSA as a circulating tumor marker for prostate cancer is a leakage of hPSA from malignant prostate tissues into the bloodstream (McCormack et al. 1995). Ellis and co-workers set in 1997 the "biological zero" for the circulating, non-prostatic hPSA to be less than 8.0 pg/ml. It takes several weeks before all hPSA is removed, or the concentration of hPSA is lowered under detection limit from blood circulation after radical prostatectomy or other strong treatments, like hormone therapy or radiation. Normally the half-life of total hPSA (protease inhibitor bound) is 2-3 days and for free hPSA 1-2 hours (Partin & Osterling 1994, 1996, Oesterling 1996, Richardson et al. 1996). It has been shown with rat experiments that the hPSA-α2-macroglobulin (hPSA-α2M) complex is removed only through the liver, while the hPSA-α1-antichymotrypsin (hPSA-ACT) complex is eliminated through the liver and kidneys (Birkenmeier et al. 1999).

hPSA is present in serum in different molecular forms, free or unbound, and bound to serine protease inhibitors. Major part of serum hPSA is bound to ACT (Lilja et al. 1991, Stenman et al. 1991). hPSA in serum is also complexed with α2M (Chen et al. 1996, Espana et al. 1996), but this complex is not detectable with current hPSA assays, due to the steric shielding effects of α2-macroglobulin to hPSA epitopes (Sottrup-Jensen 1989). This complex formation has also been shown in vitro (I, Otto et al. 1998). A minor portion of serum hPSA is complexed with other serine protease inhibitors; a complex with α1-protease inhibitor has been identified (Zhang et al. 1999). Free hPSA is usually only a minor portion of serum hPSA. Free hPSA has been suggested to be either a proform of hPSA, which is inactive or has very little activity (Kumar et al. 1997, Lövgren et al. 1997) or be formed as a consequence of internal cleavage of the peptide chain at Lys145-Lys146, causing inactivation of hPSA (Christensson et al. 1990).
No internationally accepted reference reagents are available for different forms of hPSA. Standardization of hPSA assays has been under keen discussion at international meetings (Stamey 1995, Stamey et al. 1994, Stenman et al. 1999, Brawer et al. 2001). The National Committee for Clinical Laboratory Standards has recommended the following materials: 100% free hPSA, 100% hPSA-ACT complex or 90% hPSA-ACT: 10% free hPSA, for hPSA assay calibration (National Committee for Clinical Laboratory Standards 1997). Stamey and co-workers have developed a standard for hPSA, consisting of 100% free hPSA and 90:10 hPSA (10% free hPSA and 90% hPSA-ACT complex) (Stamey et al. 1998). This so-called "Stanford" standard has been assessed as a potential WHO standard in a study by 10 units in six countries using different hPSA detection methods (Rafferty et al. 2000).

hPSA concentration in serum is also elevated in BPH. With 2D-electrophoresis it was shown that sera of BPH patients contain more internally cleaved hPSA forms than sera from cancer patients (Charrier et al. 1999). The increase of hPSA concentration in serum is also detected in prostatitis, acute or chronical inflammation of the prostate gland (Hasui et al. 1994, Potts 2000).

2.4 Human prostate-specific glandular kallikrein

2.4.1 hK2 expression and tissue distribution

hK2 is expressed mainly in epithelial cells of the prostate, which has been confirmed with immunohistochemical and in situ hybridization methods (Morris 1989, Young et al. 1996, Darson et al. 1997, 1999, IV). The amount of hK2 found in seminal plasma is about 6 µg/ml (Lövgren et al. 1999a, Frenette et al. 1999b), only ca 0.1% of the amount of hPSA (Rittenhouse et al. 1998), and the hK2 concentration in normal male serum is ~0.013 µg/L, about 2-4% of that of hPSA (Piironen et al. 1996, Klee et al. 1999, Finlay et al. 2001). hK2 concentration has also been measured from other body fluids, i.e. amniotic fluid, colostrum and milk, saliva and urine (Lövgren et al. 1999a, Magklara et al. 1999, Pannek et al. 1997). LNCaP prostate cancer cell line expresses hK2 (Grauer et al. 1996). It has been shown in LNCaP cells that hK2 expression is up-regulated in the presence of androgens and that hK2 is produced almost solely in mature form, while non-induced LNCaP cells produce prohK2 (Henttu et al. 1992, Henttu & Vihko 1993, Shan et al. 1997, Kumar et al. 2000). hK2 in seminal fluid forms a complex with protein C inhibitor (Desperthes et al. 1995, Grauer et al. 1996, Frenette et al. 1997). Fastest complex formation of hK2 is with α2M in serum, and most of the hK2 is complexed after 10 minutes' incubation with serum or plasma (Heeb & España 1998). In sera of prostate cancer patients hK2 also forms complexes with ACT (Grauer et al. 1998, Mikolajczyk et al. 1998). Complex formation between hK2 and plasminogen activator inhibitor-1, α2-antiplasmin and protease inhibitor-6 have been also reported (Mikolajczyk et al. 1999a, b, Frenette et al. 1997, Mikolajczyk et al. 1998, Saedi et al. 2001). The expression of hK2 is not limited to prostate despite previous belief, as in the case of hPSA. hK2 is expressed in
various tissues to a lesser extent than in the prostate, like breast and breast cancer cell line
(Black et al. 2000, Hsieh et al. 1997), salivary gland, thyroid (Magklara et al. 2000a) and
with RT-PCR hK2 expression has also been found in human pituitary (Clements et al.
1996). The expression of hK2 is up-regulated by androgens, progestins, glucocorticoids

2.4.2 The function of hK2

hK2 has also a typical catalytic triad of serine proteases; there is asparagine 183
conserved at its active site and therefore hK2 has trypsin-like activity (Schedlich et al.
1988, Riegman et al. 1989). Although hK2 has trypsin-like activity as does hK1, it seems
that hK2 does not possess in vitro similar kininogenase activity producing Lys-
bradykinin, but cleaves high-molecular weight kininogen producing bradykinin
(Bourgeois et al. 1997, Charlesworth et al. 1999). hK2 has been shown to activate
proPSA, a zymogen form of hPSA in vitro (Lövgren et al. 1997, Kumar et al. 1997,
Takayama et al. 1997) into mature, active hPSA, and this may be a way of regulating
hPSA activity. However, it has been reported that hK2 might not alone be able to activate
proPSA in vivo, but there are also other protease/proteases involved in this event
(Denmeade et al. 2001). The activity of hK2 is regulated by zinc, like the mouse γ-nerve
growth factor (Lövgren et al. 1999b, Pattison & Dunn 1975). The self activation by
cutting the seven amino acids long propeptide of prohK2 in order to form mature hK2 is
also thought be the way for hK2 to regulate its own activity (Mikolajczyk et al. 1997,
Denmeade et al. 2001). hK2 has been found to digest fibronectin and seminogelins I and
II in seminal fluid, but not at similar positions as hPSA (Deperthes et al. 1996). The
enzymatic activity of hK2 has been suggested to be reversibly regulated with Zn\(^{2+}\) ion in
seminal fluid (Lövgren et al. 1999b). hK2 has been shown to possess arginine-restricted
specificity, cleaving substrates at C-terminal of single or double arginines (Mikolajczyk et
al. 1998, Lövgren et al. 1999b). hK2 has also been found to activate the single-chain
urokinase-type plasminogen activator (uPA) in vitro, whereas a similar function for hPSA
has not been shown (Frenette et al. 1997). A higher amount of hK2-protease inhibitor-6
complex is found in cancerous prostate tissue than in benign tissue (Mikolajczyk et
al. 1999b). hK2 has also been found to promote the tumoral growth of prostate cancer by
degradation of insulin-like growth factor binding proteins and by increasing the
bioavailability of insulin-like growth factor (Rehault et al. 2001).

2.4.3 hK2 in prostate cancer

The value of hK2 in prostate cancer is under investigation. Sensitive serum hK2 assays
are under development, and many in-house methods are already in use (Piiroinen et al.
but commercial kits are not available yet. There are discrepancies between the hK2
assays, which are probably based on the standardization, as the cross-reactivity with hPSA has been either minimized or abolished (Becker et al. 2000b and the references therein). Methods for detection of circulating metastatic prostate cancer cells using a hK2 splice variant-specific RT-PCR assay (Slawin et al. 2000) or quantitative or other RT-PCR detection methods for hK2 and hPSA expressing cells from blood samples of healthy and prostate cancer patients have been developed (Ylikoski et al. 2001, Su et al. 2000 and the references therein). The mean hK2 concentration in sera of healthy men was 26 ng/L, while the mean hK2 concentration in sera of localized prostate cancer patients was 72 ng/L and in sera of more aggressive prostate cancer patients 116 ng/L, when measured with an ultrasensitive automated assay with a detection limit of 1.5 ng/L (Klee et al. 1999).

The expression of hK2 is higher in cancerous prostate tissue than in benign tissue (Darson et al. 1997, 1999, IV). Magklara and co-workers (2000c) obtained contrary results, a down-regulation of both hK2 and hPSA expression, when they compared cytosolic extracts from malignant prostate tissue to that of benign tissue from the same patient. Since hK2 has plasmin-like activity while activating uPA, it is suggested that hK2 could be involved in prostate cancer metastasis (Frenette et al. 1997).

2.5 Production of recombinant proteins in insect cells

Summers and Smith (1987) developed the baculovirus expression vector (BEV) system, a method which enables production of biologically and functionally active recombinant proteins in large amounts in a relatively short time. This method is also suitable for pilot-scale production of recombinant proteins (Vihko et al. 1993). The BEV system is based on the family of Baculoviridae (baculoviruses), a miscellaneous group of double-stranded DNA viruses infecting many different insect species as natural hosts (Matthews 1982). Baculoviruses infect only non-invertebrate species and the BEV system is therefore a safe method to be used in normal laboratory environment. Autographa californica nuclear polyhedrosis virus (AcNPV) is the most widely used virus with BEV system with Spodoptera frugiperda insect cell clones Sf9 and Sf21. AcNPV has a typical two-phase life span of baculoviruses, early and late. AcNPV particles enter the insect midgut cells by endocytosis or fusion, and viral DNA is uncoated at the nucleus, where it integrates into the insect genome and is ready for replication. The budding extracellular virus particles, which are formed during the early phase, infect the neighboring cells and in the late phase of the infection occluded virus particles are generated. The occluded virus particles are embedded in a matrix made of polyhedrosis protein (Rohmann 1988, Summers & Smith 1978). Occluded viruses continue to develop for 5-6 days post-infection and are detectable under light microscopy as dark polygonal bodies filling the nucleus of the infected cell. The molecular weight of polyhedrin protein is 29 kDa (O'Reilly et al. 1992). At late phase polyhedrin protein is synthetized at a high level, up to 1 mg per 1-2 million infected cells, which is about half of the total proteins. Polyhedrin protein is not necessary for the baculovirus life cycle, but in vivo occluded viruses are essential for transmission of viruses into the environment and to other insects. The BEV system takes advantage of
this phenomenon. Wild-type AcNPV DNA is co-transfected with gene of interest into Sf9 cells and the polyhedrin gene is replaced with homologous recombination in the AcNPV genome, and a recombinant virus is generated. This recombinant virus produces recombinant protein, and it also produces additional recombinant virus (Summers and Smith 1987).

Since BEV system is a eukaryotic system, the produced proteins have proper folding, formation of disulfide bonds and oligomerization (Kidd & Emery 1993). Post-translational modifications, like acylation, amidation, carboxymethylation, glycosylation, isoprenylation, phosphorylation, signal peptide cleavage and proteolytic cleavage have been reported for the BEV system (Hoss et al. 1990, Kloc et al. 1991, Kuroda et al. 1991). High expression rates of the BEV system may affect the cell's ability to modify the produced protein; this is usually seen as defects in glycosylation or phosphorylation.
3 Outlines of the present study

This work is a part of a wider research project studying the prostatic androgen regulated proteins, their structure and function. This study aims at creating recombinant serine proteases, hPSA and hK2, and characterizing these proteins as individuals. Previously this has not succeeded when starting from natural sources. Applying the knowledge gained from the recombinant hPSA and hK2 with specific tools (e.g. monoclonal antibodies) created, it was possible to study their connection to prostatic diseases.

The specific aims of this study were:

1. to generate recombinant hPSA in insect with baculovirus expression vector technology, characterize it and compare it to hPSA purified from seminal plasma.
2. to generate two polymorphic forms of hK2 as recombinant proteins, characterize them and study the allelic frequencies of the polymorphic forms.
3. to purify the hPSA isoforms secreted by prostate cancer cell line LNCaP, characterize the isoforms and compare them to recombinant hPSA and seminal fluid hPSA.
4. to study hPSA and hK2 expression in benign and prostate cancer tissue with \textit{in situ} hybridization techniques at mRNA level, and to compare the results with the expression in protein level using immunohistochemistry.
4 Materials and methods

Detailed descriptions of the materials and methods are presented in the original articles, I-IV.

4.1 Cell culture (I - III)

The insect cell line, *Spodoptera frugiperda*, Sf9, and prostate cancer cell line, LNCaP, were purchased from American Type Culture Collection and cultures according to the instructions of the supplier. The LNCaP cell line was hormone-sensitive (Vaarala *et al*. 2000) and was producing hPSA.

4.2 Recombinant serine proteases (I, II)

4.2.1 Construction of the recombinant hPSA virus

The 1464 bp long hPSA cDNA including signal and activation peptides was inserted into the EcoRI site of pVL1392 non-fusion transfer vector (Invitrogen, San Diego, CA). The orientation of the hPSA insert was confirmed by *KpnI* digestion. The transfer vector pVL1392 containing the hPSA DNA fragment was cotransfected with linearized wild-type *Autographa californica* (AcNPV) DNA, BaculoGold DNA (Pharmingen, San Diego, USA) into Sf9 cells, a modification of the calcium phosphate precipitation technique (Summers and Smith 1987). The generated virus was checked by PCR with baculovirus primers (Invitrogen, San Diego, CA, USA).
4.2.2 Construction of recombinant hK2 viruses

The coding region of hK2 cDNA was amplified from the human prostatic cancer tissue cDNA library by PCR (Clontech, Palo Alto, CA). For PCR, the N-terminal oligomer, 5'-TCCCCCGGGAGATCTCACCATGTGGGACCTGGTTTCTC-3', contained SmaI and BglII restriction sites, whereas the C-terminal oligomer, 5'-CGCTCTAGATCAGGGGTTGGCTGCGA TGGT-3', contained a XbaI restriction site in addition to the hK2 coding sequences. The hK2 cDNAs coding Arg<sup>226</sup>-hK2 and Trp<sup>226</sup>-hK2 were subcloned into the BglII/XbaI site of the pVL1392 transfer vectors. Nucleotides that differ between hK2 and PSA are shown in bold type, whereas underlining indicates identical nucleotides in the C-terminal oligomers. The PCR product was cloned into PCRII-vector (Invitrogen), and both strands of the insert were sequenced (Sanger et al. 1977). This hK2 cDNA contained T nucleotide at position 792, which led to the amino acid change from the originally reported Arg<sup>226</sup> (Schedlich et al. 1987) to Trp<sup>226</sup>, suggesting possible polymorphism in the hKLK2 gene.

Another hK2 cDNA was generated by using PCR with the N-terminal oligomer described above and a longer C-terminal oligomer, 5'-CCCGGGATCAGGGGTTGGCCTGCGATGGTCTGCCACTTC-3'; this sequence replaced T nucleotide with C at the position 792 (surrounded by asterisks), which codes for Arg<sup>226</sup> in the hK2 protein. The sequence of this cDNA was also confirmed from both directions. The transfer vectors containing either hK2 cDNA fragments were cotransfected with wild-type BaculoGold-DNA into S19 insect cells as previously described with recombinant hPSA.

4.2.3 Detections of the Arg<sup>226</sup>/Trp<sup>226</sup> polymorphism

Genomic DNA was isolated from prostate tissue obtained by prostatectomy, biopsy, or transurethral resection and from blood leukocytes (Vandenplas et al. 1984). Female and young male blood leukocyte DNA was used as control material. The 5' oligomer (5'-TTCTCACTGTGTCTCTCCTCC-3') and the biotin-labeled 3' oligomer (5'-GCGATGGTGTCTCGATCCACTTCC*G*GTATGCACC-3'); this sequence replaced T nucleotide with C at the position 792 (surrounded by asterisks), which codes for Arg<sup>226</sup> in the hK2 protein. The sequence of this cDNA was also confirmed from both directions. The transfer vectors containing either hK2 cDNA fragments were cotransfected with wild-type BaculoGold-DNA into S19 insect cells as previously described with recombinant hPSA.
4.2.4 Production of recombinant hPSA and hK2 polymorphic forms

Exponentially growing Sf9 insect cells with cell densities of 2 x 10^6 cells/ml were infected with recombinant AcNPV virus at multiplicity of infection 1. The production of recombinant serine proteases was scaled up the pilot scale from spinner flasks to 2-L (Biostat MD) and 30-L (Biostat UD 30) bioreactors (B. Braun International, Melsungen, Germany) (Vihko et al. 1993). The production of recombinant serine proteases was followed by measuring total hPSA from the culture medium with time-resolved fluoroimmunoassay kit with two monoclonal antibodies (DELFIA, Wallac, Turku, Finland).

4.2.5 Purification of recombinant serine proteases

Recombinant serine proteases hPSA and two polymorphic forms of hK2 were secreted by Sf9 insect cells to culture medium. Harvested production media were centrifuged in order to remove cell debris and concentrated with a Pellicon cassette system (10 kDa cutoff; Millipore, Bedford, MA, USA) for further purification, which was carried out at room temperature by column chromatography, using Biopilot and FPLC systems (Pharmacia, Uppsala, Sweden).

4.2.5.1 Recombinant hPSA

Concentrated recombinant hPSA production medium was dialyzed against 50 mm Na-acetate buffer (pH 6.8). In the first purification step, sample was loaded onto a fluidized bed column (Streamline 50, 5 x 100 cm, 100 ml/min, Pharmacia) containing strong cation-exchange matrix. Recombinant hPSA was eluted from the column with an isocratic salt gradient using equilibration buffer containing 0.25 M NaCl. The elute was dialyzed into 50 mM Na-acetate buffer (pH 6.8) and used for repeated cation-exchange chromatography with S-Sepharose HP (Pharmacia) column. The recombinant hPSA was eluted with linear salt gradient from 0 to 0.25 M NaCl. The fractions reacting with polyclonal rabbit anti-hPSA antibody on a slot blot were pooled and concentrated for gel-filtration chromatography with Superdex 75 (Pharmacia) eluted with 50 mM sodium phosphate buffer (pH 7.0). The fractions with the highest recombinant hPSA content were pooled and dialyzed in 50 mM sodium phosphate buffer (pH 5.8) for cation-exchange chromatography with Mono-S column (Pharmacia). The pure recombinant hPSA was eluted from the column with a linear salt gradient, 0-20mM NaCl.
4.2.5.2 Recombinant hK2 polymorphic forms

The concentrated production media, dialyzed against 50 mM sodium acetate (pH 5.5), were loaded onto cation-exchange column, Sepharose HP. The recombinant hK2 was eluted with linear salt gradient 0-250 mM. The fractions which were immunoreactive with polyclonal rabbit anti-PSA cross-reacting with hK2 were pooled and concentrated for gel-filtration chromatography with Superdex 75 column, from which the recombinant hK2 was eluted with 50 mM sodium acetate (pH 5.8) containing 150 mM NaCl. The hK2-containing fractions were pooled and dialyzed against 50 mM sodium acetate for cation-exchange chromatography through a Mono-S HR column (Pharmacia). The recombinant hK2 was eluted from the column with a linear NaCl gradient (0-200 mM).

4.2.6 Characterization of recombinant serine proteases

The purity and molecular weight of recombinant hPSA and hK2s proteins was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nonreducing PAGE. Both types of electrophoresis were carried out on a PhastSystem (Pharmacia) with gradient media 10-15 for SDS-PAGE and 8-25 for non-reducing PAGE, and the products were silver-stained (Jovin 1973, Wyckoff et al. 1977, Davis 1964, Andrews 1981, Heukeshoven & Dernick 1985).

For Western blotting, the recombinant proteins were transferred with PhastTransfer (Pharmacia) onto nitrocellulose membranes (Prieur & Russo-Marie 1988). Polyclonal rabbit anti-PSA antibody (Vihko et al. 1990) was used with a Protoblot AP system (Promega, Madison, WI, USA) to detect the expressed proteins. In both electrophoresis procedures, the recombinant hPSA and hK2 proteins were always compared with commercial hPSA purified from seminal fluid (Calbiochem, La Jolla, CA, USA). Isoelectric focusing was performed on a PhastSystem with PhastGel IEF media, pH range 3-9, and the gels were silver-stained.

The complex formation reactions between recombinant hPSA and proteinase inhibitors were performed at 37°C for different time points. Reaction mixtures were prepared, in which 10 µg of the recombinant hPSA or commercial hPSA was incubated with 40 µg of purified ACT (Calbiochem) or 200 µg of α2M (Calbiochem) in 20 mM Tris buffer containing 150 mM NaCl (pH 7.4). The reaction mixtures were analyzed by activity measurements and Western blotting of SDS-PAGE.

To study whether recombinant serine protease possessed trypsin or chymotrypsin-like activity their ability to hydrolyze specific chromogenic peptides was investigated. The reactions, performed at 37°C for one hour, were initiated by adding 50 µl of the chromogenic substrate (Pro-Phe-Arg-p-nitroanilide (pNA) for trypsin-like activity and Arg-Pro-Tyr-pNA for chymotrypsin-like activity, both from Chromogenix, Mölndal, Sweden) to 200 µl of 50 mM sodium phosphate buffer (pH 7.8) containing 100 mM NaCl and recombinant serine protease (5-15 µg). The reactions were stopped by adding 800 µl 0.6 M acetic acid, and the absorbance of the reaction mixture was measured at 405 nm (Akiyama et al. 1987).
4.3 LNCaP-PSA isoforms (III)

Hormone-sensitive LNCaP cells were grown in T-flasks, and two days after inoculation the culture medium was changed to RPMI 1640 medium, without serum, containing 10 mM of synthetic androgen 17β-hydroxy-17α-methyl-1,2,4,9,11-trien-3-one (R1881, New England Nuclear, Boston, MA, USA) to enhance hPSA production. After five days the culture medium was harvested and the amount of hPSA determined with DELFIA (Wallac).

4.3.1 Purification of LNCaP-PSA isoforms

The harvested medium was concentrated and dialyzed into 50 mM sodium acetate (pH 5.5). The concentrate was loaded onto a cation-exchange column, S-Sepharose HP, BPG 100 column, Pharmacia). Bound protein was eluted with 0.25 M NaCl. The eluate was re-dialyzed into 50 mM sodium acetate (pH 5.5) and used for repeated cation-exchange chromatographies with S-Sepharose HP (Pharmacia) column and eluted with linear salt gradient from 0 to 250 mM NaCl. The fraction reaction with rabbit anti-PSA antiserum (Vihko et al. 1990) was concentrated for gel-filtration chromatography with Sephadex 75 (Pharmacia) column eluted with 50 mM sodium acetate (pH 5.5) containing 150 mM NaCl. The fractions with the highest LNCaP-PSA content were pooled and dialyzed against 50 mM sodium phosphate, for cation-exchange chromatography with Mono-S column (Pharmacia). The LNCaP-PSA was eluted from the column with linear salt gradient of 0 to 250 mM NaCl and a step gradient of 1.0 M NaCl. The 1.0 ml fractions reacting with polyclonal rabbit anti-PSA were divided into different pools: A to D, as was done with recombinant hPSA (I).

4.3.2 Characterization of LNCaP-PSA isoforms

The purity and molecular weight of LNCaP-PSA isoforms was evaluated by SDS-PAGE and nonreducing PAGE. Both types of electrophoresis as well as Western blotting of the LNCaP-PSA isoforms were carried out as previously described with recombinant serine proteases. In both electrophoresis procedures, the LNCaP-PSA isoforms were always compared with recombinant hPSA (I) and commercial hPSA purified from seminal fluid (Calbiochem). Isoelectric focusing was performed on a PhastSystem with PhastGel IEF media, pH range 3-9, and the gels were silver-stained or blotted onto nitrocellulose membrane for Western blotting.

The enzyme activity of LNCaP-PSA isoforms was determined by their ability to hydrolyze synthetic peptide substrates for chymotrypsin-like and trypsin-like activity. The activity tests were performed as described earlier.
To determine the N-terminal amino acid sequence and processing of different isoforms of LNCaP-PSA, they were run by SDS-PAGE and transferred to the Immobilon P membrane (Millipore), cut of the membrane and analyzed with a gas-pulsed sequencer (Kalkkinen & Tilgmann 1988). The complex formation between LNCaP-PSA isoforms and protease inhibitors was tested. All reactions were performed as described earlier (I).

### 4.3.3 Structural modeling of LNCaP-PSA isoforms

The previously modeled hPSA structure (Vihinen 1994) based on crystallographically determined porcine kallikrein A structure at 2.05-Å resolution (Bode et al. 1983) taken from the protein Data Bank (entry 2pka; Bernstein et al. 1977) was used to study the consequences of the differentially processed hPSA. Professor Mauno Vihinen performed the structural modeling studies at the Institute of Medical Technology, University of Tampere, Finland.

### 4.4 Patient samples and tissue specimens (II, IV)

Tissue specimens and venous blood samples were collected from patients undergoing radical prostatectomy, biopsy or transurethral resection of the prostate, and control blood samples were collected from volunteers. The tissue and venous blood samples were stored at -70°C before DNA extraction. The hematoxylin stained specimens were examined by a pathologist to ensure that they contained both benign (normal or hyperplastic) and malignant tissue, or malignant tissue only. The patients with prostate cancer were classified according to the Tumor-Node-Metastasis (TNM) classification system (Chisholm 1988) and prostate cancer tissue specimens with the WHO histological tumor grading system (Mostofi 1980).

### 4.5 Expression of hK2 and hPSA in benign and malignant prostate tissue (IV)

#### 4.5.1 Specific hK2 and hPSA probes

The probes for in situ hybridization were a 250 bp fragment (nt 1198-1448) of hPSA cDNA (Henttu & Vihko 1989) and a 300 bp fragment (nt 820-1120) of hK2 (Schedlich et al. 1987). The fragments were amplified by PCR using oligonucleotides with T7 or SP6 binding sites. The specificities of ^32P-labeled PCR products were checked by DNA-
blotting (1 μg of hPSA and hK2 DNA per slot). The anti-sense and sense RNA probes were transcribed from PCR products by using SP6 or T7 RNA polymerases (Promega). In vitro transcription reactions with the polymerases were performed according to the manufacturer’s specifications in the presence of (α-35S) CTP.

4.5.2 In situ hybridization

The in situ hybridization reactions were performed as previously described by Chotteau-Lelievre et al. (1994) and Mustonen et al. (1998). The slides were developed after equal exposures with hPSA and hK2 probes in the dark at –20°C for 3 days. The nuclei were further stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA). The epithelial and stromal signal densities of hK2 and hPSA were measured from in situ hybridized sections with an MCID M4 3.0 digital image analyzer (Imaging Research Inc., Ontario, Canada). The epithelial signal densities were measured separately from benign and malignant epithelium using a similar method as described by Kainu et al. 1996. The silver grains were measured from 10 separate, randomly picked, 40x objective fields of epithelium and stroma. The averages of the 10 values were considered to be the respective transcript level of hK2 or hPSA in the specimen. The silver grains counted from stromal areas represented background and were subtracted from the respective epithelial values.

4.5.3 Immunohistochemistry

Sections adjacent to those used for in situ hybridization were immunohistochemically stained using specific monoclonal antibodies for hPSA and hK2, 7E7 (Vihko et al. 1990) and 151C (Höyhtyä et al. 1987), respectively. An immunoperoxidase method (DAKO StreptABCComplex/HRP Duet, Mouse/Rabbit, Dako A/S) using these antibodies was applied to the specimens. The intensity of staining was determined visually, and each specimen was assigned to one of the following categories: no staining -, low staining +; moderate staining; ++ and high/very high staining intensity ++++. The evaluation of the protein expression was done without knowledge of the respective mRNA levels.

4.6 Gene amplification study (IV)

Malignant and benign areas were dissected from paraffin blocks, and the respective genomic DNAs were isolated (Wright & Manos 1990) for a competitively differential PCR (CD-PCR)-based gene copy number analysis (Figure 3).
This method was developed by Deng and Kim (1999). They have evaluated the method for sensitivity and specificity by comparing the measured amplification by CD-PCR with that obtained by fluorescence in situ hybridization (FISH), competitively PCR and Southern blotting analysis. The concordance of this method to FISH, competitively PCR and Southern blotting analysis was 94%, 100% and 88%, respectively, when amplification of erb-2 gene was compared. The primers (5′-ATCCTCACACAAGGCTACA-3′ and 5′-AGAGGTAGGTACTGCGCTA-3′) amplifying the hKLK2 genomic sequence (AC: AF113169) between the nt 7671-8030 and the composite primers containing the respective sequences of hKLK2 at their 5′ parts, followed by the sequences for a reference gene at the 3′ parts (5′-ATCCTCACACACATGGCTACAAGGCTACA-3′ and 5′-AGAGGTAGGTACTGCTACAGGGAAGGCTACTGCGCTA-3′) between nt 928-1307 were generated. In addition, the primers (5′-CATCCTCACACATCGCTACAAGGCTACAAGGGAAGGCTACTGCTACAGGGAAGGCTACTGCGCTA-3′ and 5′-AAACAGGTGGCCTGACTGCGCTA-3′) amplifying the hKLK3 genomic sequence (AC: U37672) between nt 928-1307 and the composite primers containing the respective sequences of hKLK3 at their 5′ parts, followed by the sequences for the reference gene at 3′-parts (5′-CATCCTCACACATCGCTACAAGGCTACAAGGGAAGGCTACTGCGCTA-3′ and 5′-AAACAGGTGGCCTGACTGCGCTA-3′) were generated. A single copy gene, placental-like alkaline phosphatase (PLAP) (AC X66947), was used as a reference gene, with composite primers amplifying it between nt 74-335. A hundred nanograms of genomic DNA was amplified by PCR in 50 µl of solution containing 1X PCR buffer, 1.5 mM MgCl₂, 0.08 mM dNTP, 100 ng each of the two sets of primers and 2.5 U of the
AmpliTaq Gold enzyme (Perkin Elmer, Foster City, CA, USA). A total of 40 cycles (95°C, 60s; 58°C, 60s; 72°C, 120s) were performed. Equal aliquots of the PCR products were run in 2% agarose gel containing ethidium bromide. The gels were photographed and the optical densities of the PCR products with lengths of 262 nt (PLAP) and 360 nt (hKLK2) or 380 nt (hKLK3) were determined by a Molecular Dynamics laser scanner (Sunnyvale, CA, USA). The ratio of optical densities for the hKLK2 and PLAP PCR products was counted for each specimen. The relative gene copy number was determined by dividing the hKLK2/PLAP by the average hKLK2/PLAP in benign tissues. The gene copy number was estimated using the relative gene copy number 1.27 for hKLK2 and 1.47 for hKLK3 as a cut off point for gene duplication.

4.7 Statistical analyses (IV)

A paired sample t-test was used to compare 1) the expression levels of hK2 and hPSA and 2) the gene copy numbers of hKLK2 in benign and malignant prostate tissues. Pearson’s test was used to determine the correlation between the hK2 and hPSA mRNA levels. A Kendall two-tailed bivariant correlation test was used to compare the mRNA values and protein levels of the specimens. Statistical analyses were done with SPSS for Windows (SPSS Inc., Chicago, IL, USA).
5 Results

5.1 Characterization of recombinant hPSA (I)

The production of recombinant hPSA reached maximum of 2-4 mg/liter four days after infection of Sf9 cells with recombinant hPSA virus. The recombinant hPSA was expressed in both active and inactive form, which could be separated at the final cation-exchange chromatography step, where recombinant hPSA was eluted in five peaks. In non-reduced SDS-PAGE all five peaks contained recombinant PSA with molecular mass of 26 kDa. In native PAGE recombinant hPSA migrated in two forms, out of which the active, non-glycosylated form moved farther.

The specific activity of the pure recombinant hPSA was 98 ± 11 nmol/min per mg (mean ± SD, n=3) when synthetic peptide MeO-Suc-Arg-Pro-Tyr-pNA was used as substrate. The specific activity for commercial hPSA was 115 ± 7 nmol/min per mg (mean ± SD, n=3). Neither PSA had any detectable trypsin-like activity.

The purified active recombinant hPSA migrated as one band of 26 kDa in non-reduced SDS-PAGE, while commercial hPSA had band of a 29 kDa. In silver-stained native PAGE, active recombinant displayed one band, whereas seminal fluid PSA revealed four components. All forms of recombinant hPSA and commercial hPSA were recognized in Western blots of SDS-PAGE and native PAGE when rabbit polyclonal anti-PSA was used as antibody. Active recombinant hPSA had a major band at pl 7.7 and a minor band at pl 7.4 in silver-stained isoelectric focusing gel, while commercial hPSA existed in several isoforms with isoelectric points in a pH range from 6.0 to 7.0.

Absolute concentrations of recombinant and commercial hPSAs were determined by fluoroimmunoassay. Recoveries of recombinant and commercial hPSA were 122 ± 6% (mean ± SD, n=15) and 108 ± 20% (mean ± SD, n=25), respectively, compared to the calibrator sample of the fluoroimmunoassay kit. Recombinant hPSA formed stable complexes with ACT and α2M, as did commercial hPSA.
5.2 Characterization of the two polymorphic forms of hK2 (II)

The coding region of the hK2 cDNA amplified from the human prostatic cancer tissue cDNA library by PCR had one base difference from the earlier reported (Schedlich et al. 1987). The difference at base 792 (C vs T) resulted in production of a different amino acid at residue 226, Trp\(^{226}\) instead of Arg\(^{226}\). Sequencing DNA samples both tissue and leukocyte specimen from patients with prostatic diseases revealed a polymorphism at this base position. Out of 24 patients with prostate cancer 13 were heterozygotes (CT), 10 were homozygotes (CC) and one was a homozygote (TT). Allele frequency of the Arg\(^{226}\) was 69% and the Trp\(^{226}\) was 31%. Allele frequencies of the control blood samples, women and young males, were 67% for Arg\(^{226}\) and 33% for Trp\(^{226}\) and 66% for Arg\(^{226}\)and 34% for Trp\(^{226}\). Only homozygotes (CC) were detected when the respective region was studied in the KLK3 gene.

Arg\(^{226}\)-hK2 and Trp\(^{226}\)-hK2 were produced for the first time as mature proteins by use of baculovirus expression vector system in Sf9 cells. The molecular mass for the recombinant Arg\(^{226}\)-hK2 and Trp\(^{226}\)-hK2 was 33 kDa in reduced SDS-PAGE. An analysis of Trp\(^{226}\)-hK2 with ion-spray mass spectrometry showed an average mass of 27.4 kDa. In immunostained non-reducing PAGE Arg\(^{226}\)-hK2 showed two bands, which moved farther than the broad band for Trp\(^{226}\)-hK2. Neither form of recombinant hK2 showed any sign of glycosylation when studied with deglycosylating enzymes.

Arg\(^{226}\)-hK2 had trypsin-like activity but not chymotrypsin-like activity, its specific activity was 320.6 nmol/min per mg when measured with synthetic peptide Pro-Phe-Arg-pNA. Trp\(^{226}\)-hK2 had no detectable trypsin-like or chymotrypsin-like activity.

5.3 hPSA isoforms secreted by prostate cancer cells, LNCaP (III)

The purified LNCaP-PSA isoforms were divided in four pools into the last purification step according to elution peaks. LNCaP-PSA in all pools had a molecular weight of 29 kDa. LNCaP-PSA proved to have several isoforms. The migration of bands was different than that of seminal plasma, but rather similar to the recombinant hPSA (I) as revealed by the Western blots following native PAGE. LNCaP-PSA was heterogeneous like purified hPSA fraction from seminal plasma displaying 4-5 bands, whereas recombinant hPSA displayed 2-3 bands.

LNCaP-PSA pools had four differentially processed N-terminal sequences, correctly processed N-terminus of PSA (IVGGWEC-) and the sequences WEC- (+4 amino acids), ILSRIVGGWEC- (+4 amino acids) and SSRIVGGWEC- (+2 amino acids). Similar heterogeneity was also detected with control recombinant hPSA pools, while commercial seminal plasma PSA fraction had only correctly processed the N-terminus.

LNCaP-PSA pools possessed only chymotrypsin-like enzyme activity when measured with chromogenic peptide substrate. Pool A has the highest activity, 37 nmol/min per mg and pool B the lowest, 5 nmol/min per mg. The specific activity was lower when compared to the active recombinant hPSA (110 nmol/min per mg) and seminal plasma hPSA fraction (115 nmol/min per mg), which both had only correctly processed N-
terminus. In immunostained isoelectric focusing gels LNCaP-PSA isoforms had several isoelectric points within a pH range of 6.8-8.2.

Active isoforms from all LNCaP-PSA pools formed stable serine protease inhibitors, ACT and α2M. Complexing with α2M increased LNCaP-PSA activity in pool A twofold, pool B fourfold, and in pools C and D the activity increased slightly from the original level when measured with Arg-Pro-Tyr-pNA chromogenic peptide.

5.4 Structural modeling of LNCaP-PSA isoforms (III)

The structure of hPSA has been modeled by using porcine kallikrein as a template. The sequences have an overall sequence identity of 60%. Here the model was used to delineate properties of the differently processed forms of PSA.

The inactive precursors of serine proteases are activated by proteolytic cleavage. One of these cleavages removes the residues 14 and 15 and generates another N-terminus at residue 16. The serine proteases are highly conserved at this region, and in all known three-dimensional structures residue 16, either isoleucine or valine, is bound in a pocket in the second subdomain. This cleft is close to the catalytic site and residue 16 forms one or more hydrogen bonds with D194 from oxyanion hole. This residue is next to catalytic S195. The effect of mutations was inspected by introducing all the observed alterations to the modeled structure. The insertions and deletions at the N-terminus affect the hydrogen bond between residues 116 and D194 in the mutations. The insertions and deletions further affect the conformation activity of the catalytic site due the increased flexibility.

5.5 Analysis of hK2 and hPSA expression in benign and malignant prostate tissue (IV)

The levels of hK2 and hPSA mRNAs were compared within adjacent sections of 27 patient samples by using an *in situ* hybridization technique with °S-labeled hK2 and hPSA probes with specific activities of 3.8 x 10° cpm/µg for both probes.

hK2 and hPSA mRNAs were detected in the epithelium of both normal and BPH tissue as well as in prostate cancer tissue. This was also confirmed in protein level with immunohistochemistry. The counting of silver grains per µm² in the *in situ* hybridization analyses showed hPSA expression to be higher in benign tissues than in cancer tissues. The average expression levels (±SD) were 0.188 ± 0.95 and 0.168 ± 0.06 for benign and cancer tissue, respectively (P=0.06). The hK2 mRNA level, on the other hand, was significantly increased in cancer tissue compared to benign prostate tissue, the respective expression values being 0.203 ± 0.09 and 0.146 ± 0.06 (P<0.0005).

The results on protein level support the findings at the mRNA level: most of the specimens showed a decrease in the hPSA protein content compared to that in benign tissue. Furthermore, an increase of hK2 protein in cancer areas compared to benign areas...
was detected in a majority of the samples. However, the consistency between transcript and protein levels in some of the samples is not clear.

The number of hPSA transcripts was higher than that of hK2 in 77.3% of benign tissues, whereas the respective value in cancer tissues was only 33.3%. In BPH tissue, the mean amount of hK2 mRNA was 82% of the respective value of hPSA (P<0.003), while in cancer tissue the mean hK2 expression level was 21% higher than that of hPSA (P<0.01). There was a correlation between the hPSA and hK2 mRNA levels in both benign (r = 0.735, p<0.01) and malignant (r = 0.767, p<0.01) tissues, indicating a possibility for coordinated expression of the genes in both normal and abnormal prostate gland.

5.6 Gene amplification study for hK2 and hPSA in benign and malignant prostate specimens (IV)

A competitively differential PCR technique revealed amplification of the hKLK2 gene in cancer tissue, when the gene copy number was expected to be one in the benign tissue specimens. The average relative gene copy number was 2.3 for cancer tissue. The relative gene copy number varied from 0.81 to 1.27 in the benign specimens and from 1.62 to 2.89 in the cancer specimens. These groups of relative gene copy numbers are significantly different (P=0). The highest relative gene copy number in benign tissue was 1.27, which was chosen as a cut off point between one and two copies of hKLK2. In accordance with this, 2.54 and 3.89 represent the cut off points for three and four copies of the hKLK2 gene, respectively. Therefore, five out of seven cancer specimens contained duplication of the hKLK2 gene, and two specimens showed triplication of the gene. On the contrary, hKLK3 gene amplification was not detected in the respective cancer samples.
6 Discussion

6.1 Recombinant prostate tissue kallikreins (I-II)

Recombinant technologies have provided tools to produce substances that are difficult to obtain from their natural sources or to purify without cross-contamination of similar kind of agents/materials. Development of the BEV system has enabled recombinant protein production in insect cells with more advanced post-translational processing compared to bacteria (Summers & Smith 1987).

We have produced recombinant hPSA and two polymorphic forms of hK2 (Arg226hK2 and Trp226hK2) in insect cells using pilot-scale production techniques developed by our group (Vihko et al. 1993). The recombinant serine proteases were produced for the first time as mature enzymes. The structural similarities between classical kallikreins are 66-80% at protein level (Schedlich et al. 1987, Henttu & Vihko 1989). The hPSA assay standardization is based on hPSA purified from seminal plasma. The purity of seminal hPSA has been questioned, and contamination with serine protease hK2 has been shown (Frenette et al. 1998a, Wang et al. 1999). With recombinant hK2 polymorphic forms it was also proven to be true, while hPSA assay standardized with seminal plasma hPSA and using antibodies raised against it recognized both forms (II).

Tissue kallikreins can have different substrate specificities, like hK2 and hK7 (Lövgren et al. 1999b, Tanimoto et al. 1999). The recombinant hPSA possessed chymotrypsin-like activity and the Arg226hK2 had trypsin-like activity as predicted by their catalytic triad structures (Schaller et al. 1986, Watt et al. 1986, Schedlich et al. 1987). Part of the purified recombinant hPSA was inactive. N-terminally heterogeneous forms were found in the recombinant hPSA during the purification of the protein. The active mature hPSA was correctly cleaved during the enzyme activation process. There was either addition or deletion of amino acid residues in the N-terminus of the inactive mature hPSA enzyme. Imperfect N-terminal processing and internal peptide bond cleavages result in the inactivation of hPSA (Christensson et al. 1990, III). The active recombinant PSA formed complexes with serine protease inhibitors, ACT and α2M, alike to hPSA purified from seminal plasma. The bacterial expression systems have been used to produce recombinant proform hPSA. The inactive proform of hPSA was produced into the inclusion bodies of
the bacteria, in which case the recombinant hPSA had to be refolded and activated (Hsieh & Cooperman 2000).

Trp226hK2 was stable and inactive. The mutation of hydrophilic arginine 226 residue to the more hydrophobic tryptophan could be the reason for the inactivation of the Trp226hK2 enzyme by changing in the three-dimensional structure of protein. The inactivity of Trp226hK2 can also be a consequence of the incorrect N-terminal processing leading to deficient folding of the enzyme. A similar mutation was not found in hPSA. The active Arg226hK2 appeared to be labile. hK2 is capable of autoactivation (Denmeade et al. 2001), which could cause self-degradation during the production of the recombinant Arg226hK2. In order to increase the product yields recombinant Arg226hK2 has been produced as an inactive preproprotein (signal and activation peptides present) in *Escherichia coli* and in mammalian Syrian hamster tumor cell line (Saedi et al. 1995, Kumar et al. 1996), or as a mutated inactive proenzyme (activation peptide present), in which alanine 217 was mutated to valine (Mikołajczyk et al. 1997, Kumar et al. 1999).

### 6.2 hPSA isoforms produced by LNCaP prostate cancer cells (III)

LNCaP cells, isolated from lymph node metastasis of prostate cancer (Horoszewics et al. 1980), secrete hPSA (Horoszewics et al. 1983). We fortified LNCaP cell culture media with synthetic androgen R1881, since hPSA expression is up-regulated in LNCaP cells in presence of androgens (Henttu et al. 1992). Characterization of the purified hPSA pools showed that LNCaP cells produced N-terminally heterogeneous isoforms of hPSA, active and inactive. The molecular mass of hPSA isoforms from LNCaP cells was similar to that of the hPSA fraction purified from seminal fluid, 29 kDa. The isoelectric points of hPSA isoforms from LNCaP cells were similar to recombinant hPSA, 7.0 to 8.3. The range was also identical to pI values reported from sera of a prostate cancer patient (Huber et al. 1995). The pI-range of free hPSA from BPH patient’s sera (Huber et al. 1995) and from seminal fluid was interestingly lower, 6.6 to 7.3. hPSA isoforms from LNCaP cells formed complexes *in vitro* with serine protease inhibitors, ACT and α2M, in a similar fashion as did recombinant and seminal fluid hPSA. Recently, it has been shown that hPSA purified with a specific thiophilic interaction chromatography from LNCaP culture media also contains natural complexes with ACT and α2M (Kawinski et al. 2002). LNCaP cells also secrete hK2 (Grauer et al. 1996, Kumar et al. 2000). The presence of only chymotrypsin-like activity, when measured with synthetic peptide substrate, indicated that there was no contamination of hK2 in purified LNCaP-hPSA pools, which was also confirmed with N-terminal sequencing.

The structure of serine proteases is composed of two six-stranded antiparallel β-strands. The structures of chymotrypsin, trypsin, elastase, mast cell protease, *Streptomyces griseus* trypsin-like protein and tonin have been clarified. The protein core structure is highly conserved in the serine protease family, whereas the surface regions are highly variable with loops of different length connected to conserved regions (Greer 1990). The structure of hPSA has been modeled previously with 60% sequence similar porcine pancreatic kallikrein (Vihinen 1994, Bode et al. 1983). The molecular modeling
of hPSA isoforms from LNCaP cells showed that the deficient processing of N-terminus had an effect on correct protein folding, which had a reducing influence on the activity of the enzyme. Partially processed N-terminal regions of hPSA isoforms from LNCaP cells were not able to bind to catalytic center of hPSA. The regions were either too short to reach into the catalytic pocket, or steric shields were blocking the access of longer, more flexible forms.

6.3 hK2 and hPSA expression in benign and malignant prostate tissues (IV)

Serine proteases have been suggested to have a role in metastasis of cancers by degradation of the extracellular matrix (Mullins & Rohrlrich 1983, Torres-Rosado et al. 1993, DeClerck & Imren 1994) and having an influence of development of tumor blood vessels (Wolf et al. 2001 and the references therein). The roles of human tissue kallikreins in the different diseases are not clear yet, although over-expression or down-regulation of these genes have been reported. Especially in different forms of ovarian and prostate cancers, the expressions of KLK4, KLK9, KLK10, KLK11, KLK15 and TMPRSS2, a membrane bound serine protease, genes are up-regulated (Dong et al. 2001, Obiezu et al. 2001, Luo et al. 2001b, c, Yousef et al. 2001c, Diamandis et al. 2002, Vaarala et al. 2001). Since tissue kallikreins are secreted proteins, one can speculate that they are present in bloodstream. hPSA is the most well known and hK2 is a new biomarker for prostate diseases with traditional immunological assays. The development of sensitive assay methodology for new kallikreins, like in the case of hK6, hK10 and hK11 (Diamandis et al. 2000c, Luo et al. 2001b, Diamandis et al. 2002), enables the evaluation of their clinical value in diagnostics, follow-up or screening of different benign and malignant diseases.

We showed by in situ hybridization technique that hK2 is expressed in higher, even double, amounts in prostate cancer tissue, when compared with benign prostate tissue specimens from the same patient. The results with hPSA were reversed. Immunohistochemistry results at protein level supported the mRNA results. Previously, it has been shown that hK2 is more related to malignant prostate tumors than hPSA (Henttu et al. 1990, Darson et al. 1997, 1999). The immunoassays developed for hK2 show that hK2 concentrations are only about 1-3 % of that of hPSA in healthy males (Kwiatkowski et al. 1998, Klee et al. 1999). The physiological role of hK2 is unknown. It has been speculated that hK2 is involved in the regulation of hPSA, as it is shown that hK2 can activate the zymogen form of hPSA in vitro (Lövgren et al. 1997). The report demonstrating the antiangiogenic activity of hPSA (Fortier et al. 1999) empowered us to evaluate our results in the view that hK2 is overproduced in prostate cancer tissue in order to prevent disease progression by activating hPSA.

Gene copy numbers of KLK2 and KLK3 were analyzed using a competitively differential PCR-based system in benign and malignant prostate tissue. In the prostate cancer tissues studied, the KLK2 gene was amplified 2 or 3 times. The amplification of the KLK3 gene was not detected in the same specimens. Gene amplification is often the
reason of over-expression of genes in malignant tissue. Epidermal growth factor receptor, erbB-1, and the protooncogene erbB-2 are over-expressed in prostate cancer, and the amplification of the relative genomic content of erbB-1 in metastatic tumors and erbB-2 both in metastatic and in prostate confined tumors has been shown (Schwartz et al. 1999). High level amplification of translation initiation factor 3 gene is associated with advanced stage of prostate carcinoma (Saramaki et al. 2001). The up-regulation of KLK3 and possible other androgen-induced genes have been found in hormone-refractory prostate carcinomas after androgen receptor gene amplification (Koivisto & Helin 1999). The correlation between hK2 and hPSA expression levels indicates coordinated expression of the genes in normal and malignant prostate gland. Our results show that hPSA and hK2 give diverse information of prostate cancer development and progression.
7 Conclusions

Two members of the human tissue kallikrein gene family, hPSA and hK2, were produced as recombinant proteins using the BEV system. Recombinant serine proteases were purified and characterized. hK2 turned out to have two polymorphic forms. Recombinant hPSA had chymotrypsin-like activity, while the other form of hK2 presented trypsin-like activity and the other form of hK2 was inactive. The active hK2 was labile, obviously inactivating itself, which affected the product yields.

hPSA produced by prostatic cancer cells (LNCaP) was used as a model for the hPSA forms present in the serum of prostate cancer patients. The most significant difference between LNCaP-PSA and the hPSA fraction purified from seminal fluid was heterogeneous N-terminal processing of LNCaP-PSA. The possible structural changes in the hPSA secreted by the prostate cancer cells could affect the immunological detection and lead to discrepancies with diagnostic analyses. This should be observed when hPSA assays are developed and standardized.

*In situ* hybridization revealed that hK2 is expressed in prostate cancer tissue at significantly higher levels than in benign prostate tissue. One mechanism behind this phenomenon could be the amplification of the KLK2 gene identified from prostate carcinoma tissue in this study. On the contrary, hPSA expression was significantly higher in benign prostate tissue than prostate cancer tissue and no amplification of the KLK3 gene was detected in prostate cancer tissue. These results suggest that hK2 has a different value than hPSA in the diagnosis of prostate cancer.
References


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HUGO Gene Nomenclature Committee (HGNC) homepages, address: http://www.gene.ucl.ac.uk/nomenclature/genefamily/klkviews.html.


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