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C-PEPTIDE STRUCTURAL AND FUNCTIONAL RELATIONSHIPS STUDIED BY BIOSENSOR TECHNOLOGY AND MASS SPECTROMETRY

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To my beloved Mother & Father

“Give me strength to accept the unacceptable and help me to face reality”

ABSTRACT

Proinsulin C-peptide has a number of biological activities and receives interest focusing on the therapeutic potential as a candidate for future co-replacement therapy with insulin in type 1 diabetes. Based on conservation results from comparisons of 22 mammalian proinsulin variants, analogs were constructed for studies of phosphorylation of mitogen-activated protein kinases (MAPKs) in Swiss 3T3 fibroblasts. The results show that phosphorylation of MAPKs is promoted by the presence of conserved glutamic acid residues at three positions of C-peptide and by a helical propensity in the N-terminal segment. Degradation of C-peptide and its C-terminal pentapeptide was also studied. In serum, the bioactive pentapeptide was degraded by an aminopeptidase activity, while the full-length C-peptide was endoproteolytically degraded. Serum proteins were removed using acetone precipitation, which made it possible to detect a novel N-terminal carbamate modification identified by tandem mass spectrometry. In kidney and placenta extracts, the degradation products were identified, showing major cleavages by an N-Leu-specific endoprotease, and minor aminopeptidase-like cleavages. In attempts at purification of C-peptide binding protein(s), novel microfluidic biosensor techniques were applied and developed. Utilizing surface plasmon resonance based biosensors, binding proteins were detected in human serum and in detergent-solubilized cellular and tissue material. Components were purified by interaction with biotinylated C-peptide attached to streptavidin-coated Biacore chips. The interaction was shown to be specific by lack of binding to scrambled C-peptide. Proteins bound to the chip were eluted by micro-recovery techniques and were identified by fragment mass mappings and database searches. In all, ten proteins were identified in this manner from total extracts. Although several of the proteins are large and sticky, they show a pattern and demonstrate the power of affinity purification under simultaneous real-time monitoring of the binding. We also studied a new biosensor chip, with a large gold surface (effective area 26 mm^2), in affinity purification for enhanced protein recovery. In this manner, a 30-fold greater protein recovery than with conventional chips was demonstrated for an anti-C-peptide antibody in a one-step purification from a protein mixture. Finally, an immobilization technique was developed using an Attana biosensor instrument based on the quartz crystal microbalance technique. We demonstrate that charged molecules can be attracted to the chip surface for covalent attachment by application of a potential to the chip surface for electro-immobilization. The resulting chip was then used in biosensor capture of an anti-C-peptide anti-body, that was subsequently eluted using a two-bubble system for efficient recovery and mass spectrometric identification. All three biosensor techniques studied are reproducible through repeated cycles and provide affinity purification of proteins under real-time monitoring of the binding and elution processes.

CONTENTS

1 LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Henriksson, M., Nordling, E., **Melles**, **E**., Shafqat, J., Ståhlberg, M., Ekberg, K., Persson, B., Bergman, T., Wahren, J., Johansson, J. and Jörnvall, H. (2005) Separate functional features of proinsulin C-peptide. *Cell. Mol. Life Sci.,* submitted.

II. **Melles**, **E**., Bergman, T., Alvelius, G., Jonsson, A., Ekberg, K., Wahren, J. and Jörnvall, H. (2003) Proinsulin C-peptide and its C-terminal pentapeptide: degradation in human serum and Schiff base formation with subsequent CO_2 incorporation. *Cell. Mol. Life Sci.* **60**, 1019-1025.

III. **Melles**, **E**., Jörnvall, H., Tryggvason, S., Gemzell Danielsson, K., Ekberg, K., Tryggvason, K., Wahren, J. and Bergman, T. (2004) Degradation of proinsulin C-peptide in kidney and placenta extracts by a specific endoprotease activity. *Cell. Mol. Life Sci.* **61**, 2979-2982.

IV. Shafqat, J., **Melles**, **E**., Wiberg, D., Sigmundsson, K., Tryggvason, S., Johansson, J., Wahren, J., Öbrink, B. and Jörnvall, H. (2005) Differential protein binding to proinsulin C-peptide: a surface plasmon resonance based analysis. Manuscript.

V. **Melles**, **E**., Bergman, T., Ståhlberg, M., Thirstrup, C., Wahren, J., Jörnvall, H. and Shafqat, J. (2005) Large-surface biosensor technology for enhanced recovery in protein characterization. *J. Biomol. Tech.*, submitted.

VI. **Melles**, **E**., Anderson, H., Wallinder, D., Shafqat, J., Bergman, T., Aastrup, T. and Jörnvall, H. (2005) Electro-immobilization of proinsulin C-peptide to a quartz crystal microbalance sensor chip for protein affinity purification. *Anal*. *Biochem*. **341**, 89-93.

2 ABBREVIATIONS

Three- and one- letter codes for the 20 commonly occurring amino acids and their monoisotopic residue masses in Da.

Alanine	Ala	A	71.037
Arginine	Arg	R	156.101
Asparagine	Asn	N	114.043
Aspartic acid	Asp	D	115.027
Cysteine	Cys	C	103.009
Glutamic acid	Glu	E	129.043
Glutamine	Gln	Q	128.059
Glycine	Gly	G	57.021
Histidine	His	H	137.059
Isoleucine	Ile	I	113.084
Leucine	Leu	L	113.084
Lysine	Lys	K	128.095
Methionine	Met	M	131.040
Phenylalanine	Phe	F	147.068
Proline	Pro	P	97.053
Serine	Ser	S	87.032
Threonine	Thr	T	101.048
Tryptophan	Trp	W	186.079
Tyrosine	Tyr	Y	163.063
Valine	Val	V	99.068

CID	Collision-induced dissociation
C-peptide	Connecting peptide
ESI	Electrospray ionization
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen activated protein kinase
MS	Mass spectrometry
PC2	Prohormone convertase 2
PC3	Prohormone convertase 3
QCM	Quartz crystal microbalance
QTOF-MS	Quadrupole time-of-flight tandem mass spectrometry
RP	Reverse phase
SDS/PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TOF	Time-of-flight

3 INTRODUCTION

Diabetes is rapidly increasing in occurrence throughout many parts of the world, and it has been estimated that by 2025 there will be 300 million people affected [King et al., 1998]. Type 1 diabetes results from failure to produce insulin, while type 2 diabetes results from insulin resistance, i.e. failure to use insulin, but at least initially with maintained insulin production. Although insulin does not cure diabetes, its discovery was the first major breakthrough in diabetes type 1 treatment, largely normalizing the life of the patients for a long time. However, upon long-term treatment, type 1 diabetic patients frequently develop vascular complications, affecting especially nerves, the kidneys and the retina, leading to cardiovascular disease and the traditional late complications in diabetes.

Research now suggests that patients with type 1 diabetes are in need not only of insulin, but also of the connecting C-peptide of proinsulin in replacement therapy, and that lack of C-peptide may therefore be one of the factors behind the development of the long-term complications. With this fact in mind, we have studied structural and functional relationships of C-peptide using several methods, including biosensor technology for tracing C-peptide binding proteins. Protein separations and affinity purifications are important, including specific microcolumns on CD diskettes for selective phosphopeptide detection [Hirschberg et al., 2004], and electrocapture devices [Astorga-Wells et al., 2003] for progress in most areas of biochemical research, but additional developments are of interest to avoid limitations. Therefore we have now tested novel biosensor techniques. The microfluidic approach utilized in this thesis relies on chip-based surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) technologies. The latter provides real-time monitoring of interactions between label-free C-peptide and proteins, followed by elution and subsequent mass spectrometry (MS) in identification and structural characterization of C-peptide binding partners.

3.1 PROINSULIN C-PEPTIDE

Insulin is synthesized from its precursor preproinsulin via proinsulin in the β cells of the Islets of Langerhans in the pancreas [Steiner et al., 1967]. Proinsulin is converted by a process of enzymatic cleavages to insulin and C-peptide in the granules (Fig. 1). The two endopeptidases, prohormone convertases 2 and 3 (PC2 and PC3), cleave the proinsulin molecule at sites marked by pairs of basic amino acids. PC3 cleaves at the Arg31-Arg32 B/C chain junction site of proinsulin and PC2 cleaves at the Lys64-Arg65 C/A junction site [Clark, 1999]. Carboxypeptidase H removes the basic C-terminal residues at each site. When the cleavage at both sites is complete, C-peptide and insulin are produced, stored in granules, and released into the circulation in equimolar amounts [Bonser and Garcia-Webb, 1984].

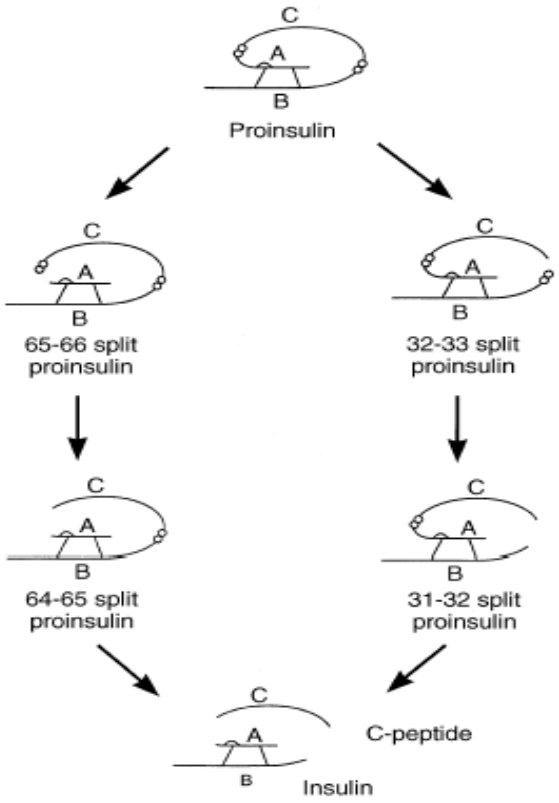

Figure 1. Processing of proinsulin to insulin and C-peptide (adapted from Clark, 1999).

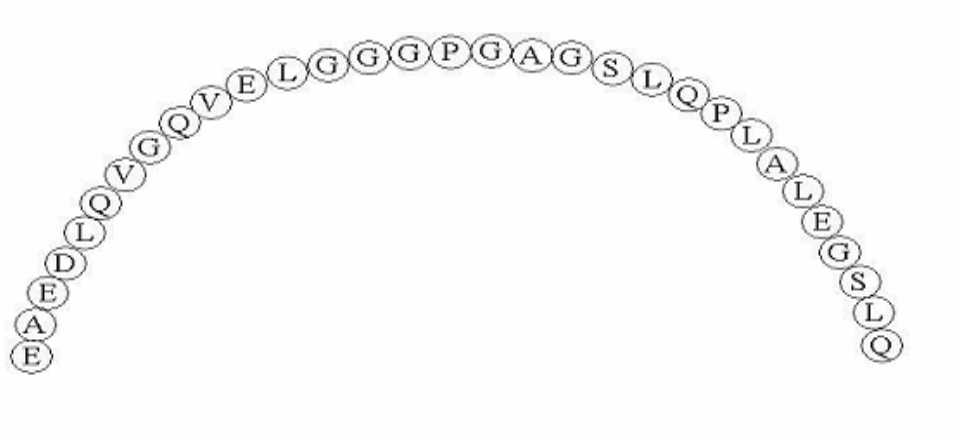

Figure 2. The human proinsulin C-peptide 31-residue amino acid sequence.

The discovery and purification of insulin was the result of the collaborative work of Banting and Best, later assisted by Collip, and all in the laboratory of Macleod in 1921-22. Insulin was tested on diabetic patients 1922, revolutionizing the treatment of diabetes mellitus [Bliss, 1982]. Ever since then, insulin has been used for treatment of diabetic patients saving millions of lives. Insulin is today a well-established and well-described hormone. C-peptide was purified and characterized in the late 1960-ies by Steiner [Steiner, 1967; Clark et al., 1969]. It is a highly acidic 31-residue peptide with a molecular weight of 3020 Da [Steiner et al., 1967; Ko et al., 1971] (Fig. 2). An established function of C-peptide is to assist correct folding and disulfide pairing of the A and B segments of proinsulin, which is necessary for generation of mature insulin after cleavage [Steiner, 1978]. For a long time this was thought to be the only function of C-peptide. However, other studies showed that C-peptide also has physiological activity by itself [Johansson et al., 1992; Wahren et al., 1994], gradually leading to the present situation, reflected in a recent review [Marques et al., 2004]: "C-peptide, much more than a byproduct of insulin biosynthesis". During the last decade, many studies have shown that C-peptide is a biologically active peptide [Johansson et al., 2002; Wahren and Jörnvall, 2003]. The physiological function is ascribed to the secreted C-peptide and interest now focuses on the therapeutic potential in

prevention of type 1 late diabetic complications [Wahren, 2004; Wahren et al., 2004].

Upon administration of C-peptide to diabetic patients, improvements are seen regarding nerve conductivity [Johansson et al., 1996], renal function and glucose utilization [Johansson et al., 1992]. However, the mechanisms underlying the beneficial effects of C-peptide are unknown. At the molecular level, C-peptide stimulates phosphorylation of mitogen activated protein kinases (MAPKs) in mouse embryonic fibroblast Swiss 3T3 cells ([Kitamura et al., 2001]; Paper I) in mouse lung capillary endothelial cells [Kitamura et al., 2002], and in human renal tubular cells [Zhong et al., 2004]. Requirements for protein kinase C, phosphoinositide 3-kinase, pertussis toxin-sensitive G-protein [Kitamura et al., 2001; Zhong et al., 2004], and other factors [Ido et al., 1997] have also been shown, as well as insulinomimetic effects [Grunberger et al., 2001; Grunberger and Sima, 2004]. Several studies suggest that C-peptide exerts its effects through a G-protein coupled receptor [Ohtomo et al., 1998; Rigler et al., 1999; Li et al., 2001], but other receptors have also been implied [Johansson et al., 2002], as well as mediation via non-chiral mechanisms [Ido et al., 1997]. However, a defined receptor has not been reported. The structure of C-peptide is unordered under physiological conditions, but the N-terminal part (residues 1-11) may adopt an α-helical conformation in the presence of high concentrations of trifluoroethanol (TFE) [Henriksson et al., 2000]. Other evidences suggest that the C-terminal pentapeptide of C-peptide has an activity similar to that of full-length C-peptide in displacing C-peptide bound to a cell surface [Rigler et al., 1999], in molecular assays [Ohtomo et al., 1998; Shafqat et al., 2002] and in stimulating glucose utilization in diabetic rats [Sato et al., 2004]. Importance of Glu27 for binding to cell surfaces has been described [Pramanik et al., 2001] and the N-terminal Glu residue of C-peptide has been ascribed some role in receptor interactions [Bourguignon et al., 1994].

Despite all the beneficial effects of C-peptide, and in the absence of any study showing side-effects or harmful actions, its role as a conventional

hormone is not yet universally accepted. One of the reasons for this is that C-peptide shows wide species variability, a property atypical of structures in functional interactions, such as hormones. In Paper I, the sequence variability of proinsulin was studied in different species, from Atlantic hagfish to human. As already known, the insulin A and B chains are highly conserved. In contrast, C-peptide has only two residues highly conserved (>70%). We constructed novel C-peptide analogs with different features for measurement of stimulation of phosphorylation of ERK1/2 in Swiss 3T3 fibroblasts (Paper I). In the subsequent two Papers (II and III) we studied the stability and degradation of C-peptide, aiming at a better understanding of its degradation mode in tissues. In Paper IV, differential protein binding to C-peptide was studied by SPR measurements with serum and with cellular/tissues extracts in a Biacore 3000 instrument. Using reversible attachments to chips with immobilized C-peptide, or as a negative control scrambled C-peptide, proteins recovered were identified with mass spectrometry. In Paper V and VI, new biosensor instruments and immobilization techniques are introduced. Capabilities for enhanced protein affinity purification (Paper V) and novel immobilization advantages (Paper VI) are demonstrated.

3.2 BIOSENSORS

A biosensor is a device that uses label-free techniques to detect other biologically active molecules. Typically, the detector molecule must be connected to a sensor that can provide real-time monitoring. Biosensors based on biological materials are now used in a wide variety of disciplines, including food industry, medicine, and environmental science. It is becoming increasingly important for scientists in these and other fields to understand the different types of biosensors that can be used. The principles behind both SPR based and QCM based biosensors as well as their advantages and limitations are discussed below.

3.2.1 Surface plasmon resonance

The SPR technique relies on a phenomenon that occurs when polarized light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media of different refractive index: the glass of a sensor surface with high refractive index and a buffer with low refractive index. A wedge of polarized light, covering incident angles, is directed toward the glass face of the sensor surface and reflected light is detected within the system. Electric field intensity, known as an evanescent wave, is generated when the light strikes the glass. This evanescent wave interacts with, and is absorbed by, free electron clouds in the gold layer, generating electron charge density waves called plasmons and causing a reduction in the intensity of the reflected light. The resonance angle at which this intensity minimum occurs is a function of the refractive index of the solution close to the gold layer on the opposing face of the sensor surface (Fig. 3) [Kretschmann, 1968; Homola, 2003]. SPR biosensing has been demonstrated in the past decade to be a very powerful technique. This technique has a unique capability to quantitatively measure the interactions of many biomolecules with ligands and membranes, including protein-ligand, protein-protein, protein-DNA and protein-membrane binding. SPR biosensing provides means, not only for detection of these interactions and quantification of their binding constants (Fig. 4), but also for ligand fishing, i.e. affinity purification of binding proteins at a very sensitive level which is label-free and provides real-time monitoring [Malmqvist, 1993; Cooper, 2003; Tsoi and Yang, 2004]. In this thesis, a Biacore 3000 instrument from Biacore AB [Karlsson, 2004], and a Vir Biosensor chip [Thirstrup et al., 2004], both based on the SPR technique, have been used.

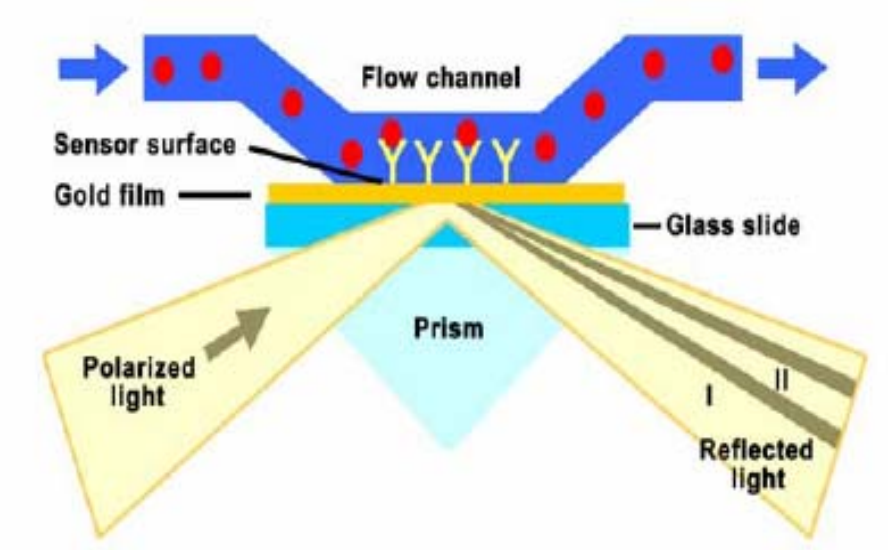

Figure 3. Principle for an SPR-based biosensor instrument. To a glass slide with a thin gold coating, a ligand is attached via a dextran layer and the assembly is mounted on a prism. Light passes through the prism, is reflected by the gold, and passes back through the prism to a detector. Changes in reflectivity versus angle or wavelength give a signal that is proportional to the volume of biomolecule analytes bound to the ligand near the surface. A flow cell allows solutions above the gold surface to be rapidly changed (adapted from Biacore).

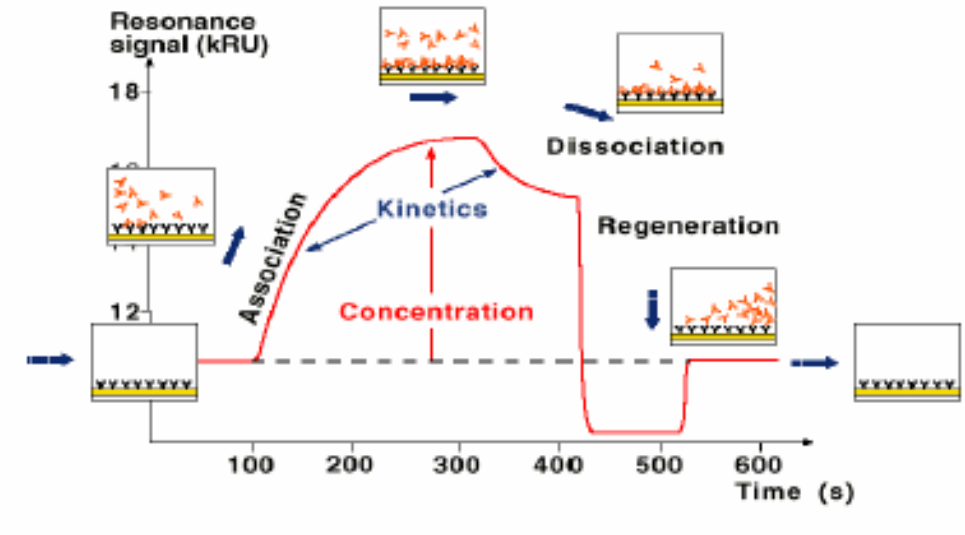

Figure 4. SPR sensogram showing the response versus time. To the surface with immobilized ligand, buffer injection is started at time 0. At 100 sec, a solution containing the analyte is injected. At 300 sec, the flow cell is washed with buffer, and at 420-520 sec, the surface is washed with regeneration buffer (adapted from Biacore).

3.2.2 Quartz crystal microbalance

Crystals that obtain a charge when compressed or twisted are said to be piezoelectric. The word piezo is Greek for "push". The piezoelectric pressure effect in quartz crystals was first discovered in 1880 by the brothers Pierre and Jacques Curie. A year later, the brothers discovered the inverse phenomenon, that the application of a voltage to a piezoelectric material will deform it. Consequently, the application of an alternating current can result in an oscillation in a piezoelectric material. Shearing a piezoelectric material along specific crystalline directions and sandwiching it between two electrodes, can control these oscillations [Aastrup, 1999]. Important developments have been good crystal stability through the use of electric resonators and room-temperature stable AT-cut crystals [Lack, 1934]. QCM is a simple, cost effective, high-resolution mass-sensing technique, first used for mass-sensing in 1959, when Sauerbray reported a linear relationship between the frequency decrease of an oscillating quartz crystal and the bound elastic mass of deposited metal [Sauerbrey, 1959]. Early chemical applications of QCM were to measure mass-binding of gas-phase molecules to the quartz surface. These instruments represented some of the earliest chemical sensors for moisture and volatile organic compounds, environmental pollutants, and gas-phase molecules in chromatography detectors [Marx, 2003]. In the 1980s, solution-based QCM developed as a new oscillator technology advanced to measure changes in frequency that could be related to changes in viscosity and density in highly damping liquid media. The recent success of the QCM technique is due to its ability to measure mass changes associated with liquid-solid interfacial phenomena in a sensitive manner [Marx, 2003]. In this thesis, we demonstrate the capability of the QCM technique for affinity purification of proteins in an Attana microfluidic biosensor instrument (Fig. 5). The novel feature is immobilization of acidic peptides, such as C-peptide, using an electric field-assisted approach (Paper VI).

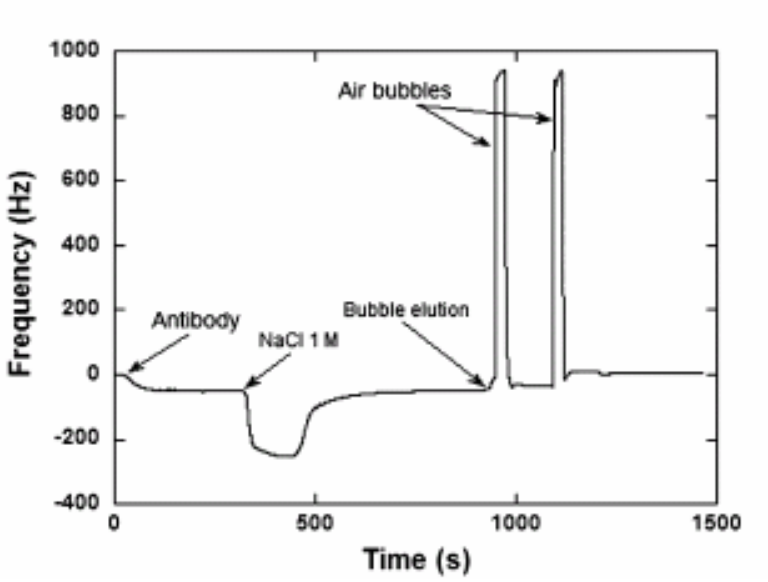

Figure 5. Frequency shift profile of the QCM-based biosensor, showing binding of the antibody to the immobilized C-peptide, washing with 1 M NaCl, and elution of the antibody by 5% formic acid using a two-bubble system (Paper VI).

3.3 MASS SPECTROMETRY

Mass spectrometry is a powerful analytical technique for protein characterization that is widely used in industry and academia for both routine and research purposes [Tyers and Mann, 2003]. Its uses include accurate molecular weight measurements, verification of amino acid substitutions, post-translational modifications, chemical modifications, protein cleavages, sequence confirmation, de novo characterization of peptides and identification of proteins by database searching of masses or sequences of proteolytic fragments [Henzel et al., 1993; Pappin et al., 1993; Mann and Wilm, 1994; Larsen and Roepstorff, 2000; Meri and Baumann, 2001; Aebersold and Mann, 2003], monitoring of enzyme reactions [Mattei et al., 2004] and studies of protein folding by H/D exchange and protein-ligand complex formation [Garcia et al., 2004; Pantazatos et al., 2004; Spraggon et al., 2004].

A mass spectrometer can be divided into three fundamental parts: the ion generator, the mass separator and the detector. In the ion generator,

sample molecules are transferred to the gas-phase as positive or negative ions. The ions are then separated according to their mass-to-charge ratios (*m/z*). Finally, the separated ions are detected and the data stored for presentation in the format of a *m/z* spectrum. The separator and detector of the mass spectrometer, and often also the ion generator, are maintained under high vacuum to give the ions a reasonable chance to travel from one end of the instrument to the other.

Many ionization methods are available and each has advantages and disadvantages. The ionization method of choice depends on the type of sample under investigation and the mass spectrometer technology. In this thesis, electrospray ionization (ESI) [Fenn et al., 1989; Wilm and Mann, 1996] and matrix-assisted laser desorption/ionization (MALDI) [Karas et al., 1987; Karas and Hillenkamp, 1988] are utilized. Both ESI and MALDI employ low energy for ionization of the molecules and are thus compatible with structurally labile compounds. This principle makes these ionization processes suitable for large biomolecules like proteins and peptides. In MALDI, the proteins or peptides are mixed with a light-absorbing matrix. The matrices most used are α-cyano-4-hydroxycinnamic acid, sinapinic acid and 2,5-dihydroxybenzoic acid. In addition, ESI has a low tendency to disrupt non-covalent interactions. Consequently, it can be used for studies of protein-protein and protein-ligand interactions. For ESI, the sample is dissolved in a volatile solvent and sprayed from a capillary needle held at high potential.

The main function of the mass analyzer is to separate the ions formed in the source based on their *m/z* ratios. There are a number of mass analyzers currently available, the better known include quadrupoles, time-of-flight (TOF) analyzers, magnetic sectors, and both Fourier transform and quadrupole ion traps. These mass analyzers have different features, including the *m/z* range that can be covered, the mass accuracy and the achievable resolution.

The detector monitors the ion current, amplifies it, and then transmits the signal to the data system where it is recorded in the form of mass spectra.

The relative intensities of the ions are plotted against their *m/z* values to show the number of components in the sample, the molecular weight of each component, and the relative abundance of the components in the sample. Common detectors are the photomultiplier, the electron multiplier and the microchannel plate.

Tandem mass spectrometry, using collision-induced dissociation (CID) is efficient for sequence determination of peptides [Hunt et al., 1986; Papayannopoulos, 1995]. Alternative methods, such as surface-induced dissociation [Grant and Cooks, 1990; Dongre et al., 1996] and electron capture dissociation [McLafferty et al., 1998; Kelleher et al., 1999] also exist, but are not as commonly used as CID. The principle for tandem mass spectrometry in amino acid sequence analysis is selection of a peptide precursor ion using a first mass analyzer and then directing this ion into a collision cell held at a slight pressure of an inert gas such as argon. Collisions occur between the precursor ion and the gas atoms/molecules, generating product ions or fragments of the original peptide for which the masses are determined in a second mass analyzer. Under conditions of low-energy collisions, small neutral losses and cleavage at the peptide bonds dominate the fragmentation pattern. From the spectrum of fragment masses, the amino acid sequence of the peptide can be deduced [Papayannopoulos, 1995].

3.3.1 Combining biosensor technology and mass spectrometry

Combining protein function with identity is important for research in protein science. This approach of functional proteomics is constantly growing. The first attempt to combine biosensor instrumentation with MS analysis was preformed by Nelson and co-workers [Nelson and Krone, 1999; Nelson et al., 2000]. In their procedure, the SPR sensor chip used in monitoring the interaction was detached from the biosensor instrument with the molecules still bound to the chip surface and MALDI-TOF-MS was used to directly determine the molecular mass of proteins remaining on the sensor chip surface [Nelson et al., 1999; Nedelkov and Nelson,

2000]. This approach is easy to apply, necessitating only minor modifications of the target plate for mass spectrometric analysis. However, it turns out to have many limitations such as a low amount of protein accessible for MALDI-TOF-MS and poor reusability of the chip. Since the MALDI matrix is applied directly to the chip, the chip is partly destroyed. In addition, determination of intact molecular masses by MALDI-TOF-MS cannot be considered a general method for protein identification, because of poor mass accuracy and the presence of unknown post-translational modifications.

To be able to choose the best analytical tool for further analysis, bound molecule(s) must be removed from the chip and preferably also without destruction of the chip. Sönksen and colleagues reported about a micro-recovery method to elute the bound molecule between two air bubbles "sandwich elution" [Sönksen et al., 1998]. This elegant method provides recovery without dilution of the bound material. In this thesis, a modified version of this two-bubble method was used. However, the eluted proteins must be enzymatically digested for proper identification by MS or tandem MS, and in this process, the samples are partly lost. To overcome this problem and accumulate sufficient material, the two-bubble elution can be repeated since this procedure is non-destructive to the sensor chip.

Another elegant method was developed by Natsume and colleagues for "on-chip" digestion of binding proteins and uses a solution containing an enzyme that is "parked" in touch with the chip surface while the SPR detector monitors the decrease in binding during the proteolytic process. Peptides generated are subsequently eluted and trapped on a reverse phase (RP) capillary column at the outlet of the flow cell. The column is connected to a microelectrospray interface and the peptides are analyzed by tandem MS [Natsume et al., 2000].

Even though the Sönksen and Natsume methods are very elegant, each method has its limitations. The two-bubble elution is a more easily applied approach and also used as a standard technique in the Biacore system. However, there are limitations in terms of recovery of proteins,

particularly of those that dissociate very fast [Mattei et al., 2004]. Even though there is a recovery kit provided by Biacore, the amount of protein recovered is generally in the subpicomole range. Furthermore, the kit does not provide real-time monitoring of the binding event [Biacore, 2003]. Having these and other limitations in mind, we wished to improve existing biosensor technology and introduce new methods.

In general, there is a bright future for biosensor/MS combinations [Merchant and Weinberger, 2000], and an increasing number of commercially available instruments promote the development of novel sensor surfaces, immobilization techniques and attachment chemistries, to allow any ligand-analyte complex to be screened [Cooper, 2002]. In Paper V, a novel large-surface biosensor chip based on the SPR technique with the optical system integrated into the chip and with an effective gold surface of 26 mm^2 was introduced [Thirstrup et al., 2004] (essentially as in Fig. 3, but without the prism). We utilized the two-bubble system (modified from [Sönksen et al., 1998]) for elution. In Paper VI, another biosensor principle in an instrument from Attana AB employing the QCM technique was introduced. This biosensor has novel advantages. One is that the sensor surface can be manipulated by application of an electric field, which attracts positively or negatively charged molecules to the sensor surface for covalent immobilization depending on the polarity of the voltage applied. We demonstrate this by using C-peptide which is highly acidic (net charge –5 and pI 3.3). We also show the capacity of the chip for affinity purification of proteins using the two-bubble system.

4 AIM OF THE THESIS

To find and apply methods for study of C-peptide structural and functional relationships, aiming at a better understanding of C-peptide stability and the influence of C-peptide on protein phosphorylation. In addition, to improve biosensor technology regarding protein-binding capacity and ligand immobilization in affinity purifications.

5 MATERIALS AND METHODS

5.1 PHOSPHORYLATION ASSAY

Species variants of proinsulin were assembled from databanks and aligned using the software CLUSTAL W [Thompson et al., 1994]. For secondary structure analysis of the peptides, circular dichroism was used. The mouse embryonic fibroblast cell line Swiss 3T3 was stimulated with C-peptide and different analogs. The protein content in the stimulated cells was determined using the BCA protein assay. The resulting samples were subjected to SDS/PAGE on 12% Tris/glycine gels or NuPAGE 10% Bis-Tris, followed by blotting to 0.2μm porosity PVDF membranes. The phosphorylated proteins were detected using specific antibodies. The densities of appropriate bands reflecting phosphorylated proteins were analyzed using a Bio-Rad GS-710 imaging densitometer and Quantity One software. Further experimental details are described in Paper I.

5.2 DETERMINATION OF C-PEPTIDE STABILITY AND DEGRADATION

In Paper II, human serum was diluted and mixed with synthetic C-peptide and C-terminal pentapeptide for incubation at 37°C. Aliquots were withdrawn directly and after specified times. For samples from C-peptide incubations, acetone precipitation was carried out to a final concentration of 70% (v/v) acetone. The samples were acidified by TFA (pH 2), followed by centrifugation at 10 000 rpm for 10 min. They were then filtered through a 0.2 μm membrane filter (NanoSep, Pall Gelman Laboratory) and submitted to RP-HPLC. Fractions were collected manually in Eppendorf tubes, completely dried under a stream of nitrogen and dissolved in 60% acetonitrile containing 1% acetic acid for nano-electrospray mass spectrometry.

For Paper III, mouse kidneys were recovered from anesthetized animals (approved by the local ethics committee) and stored at -70°C. Kidneys

were disintegrated in 100 mM HEPES buffer, pH 7.1, using a Potter Elvehjem homogenizer. Homogenate aliquots of 50 µl were stored at -70°C. Human placenta (immediately frozen) was obtained from Karolinska University Hospital (approved by the local ethics committee). After thawing, 14 g was disintegrated in 100 mM HEPES buffer, pH 7.1, using an Ultraturrax T25 mixer, resulting in 5 ml homogenate that was stored in 500 µl aliquots at -70°C. For degradation studies, human and mouse C-peptides (26 nmoles; PolyPeptides), were incubated with a 10-fold dilution of the crude homogenates in 100 mM HEPES buffer, pH 7.1, at 37°C. As blank, a 10-fold dilution of kidney or placenta homogenate in the same buffer was used. Aliquots corresponding to 4.3 nmoles C-peptide were withdrawn at specific time points. The aliquots were acidified (pH 2) by addition of TFA, followed by centrifugation at 10 000 rpm for 10 min. After filtration through a 0.2 µm membrane filter (NanoSep, Pall Gelman Laboratory), the samples were analyzed by RP-HPLC. Fractions were collected in Eppendorf tubes, completely dried under a stream of nitrogen and dissolved in 60% acetonitrile containing 1% acetic acid for nano-electrospray mass spectrometry. Further experimental details are described in Papers II-III.

Mass spectra were recorded using a Q-TOF mass spectrometer (Waters) equipped with an orthogonal sampling ESI-interface (Z-spray, Waters), and metal-coated nano-ESI needles (Proxeon) with a spraying orifice of about 5 µm and a flow rate of approximately 20–50 nl/min at a capillary voltage of 0.8–1.2 kV. For acquisition of CID spectra, the collision energy was optimized in the range 30–80 eV, with argon as the collision gas.

5.3 BIOSENSOR ANALYSIS

5.3.1 Cells and preparation of cellular extracts

Human embryonic kidney 293 cells were obtained from DMSZ GmbH, L6 cells (rat skeletal muscle myoblast cell line) were from American Type Culture Collection (Rockville, MD), human renal tubular cells with proper permit and human serum, were obtained from Karolinska Hospital.

Human umbilical venous endothelial cells were from Cambrex Bio Science (Rockland, USA). Mouse kidneys were recovered from anesthetized animals and stored at -70°C. The cells were cultured at 37°C in a humid CO_2 incubator (92% air, 5% CO_2) grown to 95-100% confluence. Cells grown on culture dishes were washed twice with PBS, either serum-starved for 2 h or directly scraped off and centrifuged at 4ºC for 4 min at 2000 x g. The cell pellets were dissolved in different detergents, Brij-58, CHAPS, digitonin, chenodeoxycholate, sodium cholate, deoxycholate, or dodecyl maltoside, at concentrations from 1 x critical micelle concentration (CMC) to 6 x CMC. Solubilization was carried out at different cell/solute ratios during 20-60 min at 4ºC, with addition of a complete protease inhibitor cocktail (Boehringer Mannheim) and endonuclease (Sigma). Tris-buffered saline (TBS) was used as solute. The cellular extracts were centrifuged at 100 000 x g for 20 min to 1 h, and the supernatant was used for SPR studies. Further experimental details are described in Paper IV.

5.3.2 Immobilization of biotinylated peptides on streptavidin-coated sensor chips

Streptavidin-coated sensor chips (Biacore AB) were used for immobilization of biotinylated peptides. Briefly 0.02 mg/ml of biotin-labeled peptides in running buffer containing 0.01 M Tris/HEPES, pH 7.4, 3 mM EDTA, 0.005% surfactant P20, 0.15M NaCl (TBS/HEPES) were applied to an SA-chip. Guanidine-HCl was used for regeneration of the sensor chip surface. SPR measurements and micro-recovery was carried out utilizing Biacore 2000 and 3000 instruments. The samples were allowed to pass over the chip surface at 10 µl/min and 25^{o}C. Recorded data were scale transformed, background subtracted, evaluated and plotted using BIA evaluation 3.1 software. Micro-recovery was performed using a program with 22 recovery cycles, each with 4 µl 1% TFA.

5.3.3 Immobilization of biotinylated C-peptide on neutravidin coated sensor chips

For binding of the affinity ligand to the chip, neutravidin was first adsorbed to the chip gold surface. For the coating process, the chip was initially rinsed in a stream of water for a few sec to remove dust and particles. It was then submerged in hypochlorite solution for 4 min under stirring, rinsed in a stream of water for a few sec, and dried. A biosensor instrument (Cobi R100, Vir Biosensor, Taastrup, Denmark) was prepared with a cleaning chip positioned in the flow cell and the pump was adjusted to 300 µl/min. The tubing and flow cell were washed with NaOH and water. The gold chip was then inserted into the instrument. The pump was started with the inlet tube submerged in calibration solution containing glycerol. The flow cell was rinsed with NaOH and water to remove glycerol. The gold surface of the chip was equilibrated with sodium acetate (pH 5), after which 10 µg neutravidin/ml in sodium acetate (pH 5) was injected. Unbound neutravidin was removed by washing with sodium acetate (pH 5) complemented with 0.005% Tween. After this treatment, the chip was washed with 10 mM TBS, pH 7.4, and biotinylated C-peptide at 20 µg/ml in TBS was pumped into the flow cell. Finally, the chip was washed with TBS containing 0.005% Tween 20.

5.3.4 Electro-immobilization of C-peptide to a quartz crystal microbalance sensor chip

In Paper VI, a QCM based biosensor instrument (Attana 80, Attana AB) was used. An Attana carboxyl chip was treated according to the manufacturer's instruction and docked into the instrument. The carboxyl groups on the surface were activated with a mixture of equal volumes of hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in water. After activation of the chip surface, a potential of - 4 V was applied between the sensor surface and the counter electrode. This provides a positive charge to the surface and thereby prepares it for binding the negatively charged C-peptide to the chip surface. The C-peptide solution was injected for covalent immobilization of the peptide

N-terminus to the activated carboxyl groups of the chip surface. The potential was then set to zero and the chip surface was deactivated with ethanolamine. To remove non-specifically bound C-peptide, the surface was washed with guanidine-HCl. The solvent carrying C-peptide was varied among different ionic strengths, as was also the potential between the electrodes. Further experimental details are described in Paper VI.

5.4 AMINO ACID ANALYSIS

The recovery after elution of chip-bound protein was evaluated by amino acid analysis in a ninhydrin-based analyzer (Biochrom 20, Amersham Pharmacia Biotech) of aliquots hydrolyzed for 24 h at 110^{o}C in sealed, evacuated tubes with 6 M HCl containing 0.5% (w/v) phenol.

5.5 SDS/PAGE, IN-GEL DIGESTION AND PEPTIDE MASS FINGERPRINTING

The fractions collected (papers IV, V and VI) were analyzed by SDS/PAGE, using 12 % Tris-glycine gels and silver-staining. The protein bands were excised manually from the silver stained SDS/PAGE gels and digested with trypsin using a MassPREP robotic protein handling system (Waters). Tryptic peptide extracts from in-gel digestion were mixed at a 1:1 (v/v) ratio with a saturated solution of α-cyano-4-hydroxycinnamic acid in 75% aqueous acetonitrile containing 0.1% TFA, and dried on a standard target plate followed by MALDI-MS (Voyager DE Pro, Applied Biosystems). The resulting peptide mass maps were submitted to database searches using the MS-fit program www.prospector.ucsf.edu.

6 RESULTS AND DISCUSSION

6.1 FUNCTIONAL FEATURES OF PROINSULIN C-PEPTIDE (PAPER I)

The capability of C-peptide to induce phosphorylation of mitogen activated protein kinases (MAPKs) was studied using 3T3 cells. Synthetic C-peptide analogs designed from conclusions based on the sequence conservation pattern in 22 mammalian proinsulin variants were prepared. In these, when the internal Glu residues at positions 3, 11 and 27 were replaced with Ala residues, no significant phosphorylation was detected, while human C-peptide produced a MAPK phosphorylation that was 1.2-3.7 times the background phosphorylation level. These findings are compatible with activity studies of C-peptide. Two other analogs were designed to test the effect of altering the helical propensity in the N-terminal part of C-peptide on stimulation of phosphorylation. The introduction of Ala residues at positions Asp4, Val7, Gly8 and Val10, which promotes the α-helical structure of the intact C-peptide, produced the same increase of the phosphorylation as did the intact C-peptide. In contrast, the introduction of Pro residues at positions Asp4, Val7, Gly8 and Val10, which disturbs α-helix formation, resulted in no stimulatory effect on phosphorylation.

In conclusion, efficient phosphorylation of MAPKs is promoted by the presence of the conserved Glu residues at positions 3, 11 and 27, and the propensity to form an α-helix in the N-terminal segment of the peptide. This suggests a tripartite nature of C-peptide, in which the terminal sections, including the conserved Glu residues and specific secondary structures, may be involved in functional interactions, while the variable mid-section appears to form a joining segment. These results extend the knowledge about critical residues in C-peptide and suggests that for total effects in cellular systems, the entire C-peptide is important, which is compatible with complex or multiple effects of C-peptide.

6.2 PROINSULIN C-PEPTIDE AND ITS C-TERMINAL PENTAPEPTIDE: DEGRADATION IN SERUM AND N-TERMINAL CARBAMATE MODIFICATION (PAPER II)

The degradations of proinsulin C-peptide and its C-terminal pentapeptide in serum were analyzed by RP-HPLC and QTOF-MS. The aim was to identify the degradation mode of the full-length C-peptide and the biologically active C-terminal pentapeptide segment. The peptides were incubated in serum and aliquots were recovered at specific time points for analysis by RP-HPLC. The results reveal degradation of both peptides, but by different modes and rates. The short pentapeptide was found to be more rapidly degraded than the full-length C-peptide, and by an exopeptidase activity (aminopeptidase). The full-length C-peptide was found to be differently degraded suggesting an endopeptidase activity. Protection of the pentapeptide N-terminus by acetylation results in a prolonged half-life. Serum proteins were removed before RP-HPLC using acetone precipitation which made it possible to detect the native C-peptide and identify a novel N-terminal carbamate modification, as shown by tandem MS. This structural modification did not occur when the C-peptide was N-terminally blocked by a preceding acetylation or when the N-terminal residue was not Glu.

In conclusion, this study identifies different degradation modes for full-length C-peptide and its biologically active C-terminal pentapeptide segment, characterizes the aminopeptidase degradation of the pentapeptide, and identifies a novel N-terminal carbamate modification.

6.3 DEGRADATION OF PROINSULIN C-PEPTIDE IN KIDNEY AND PLACENTA EXTRACTS (PAPER III)

The second study established low degradative activities in serum. However, the major C-peptide degradation is known to be in the kidney. Therefore, in Paper III, we determined the mode of degradation of C-peptide in kidney and, for comparison, placenta extracts, identifying the degradation products by use of RP-HPLC and QTOF-MS. Eight

degradation products were identified in kidney and seven in placenta extracts. More than half of the fragments determined were found to be the same in both extracts. C-peptide was shown to be degraded by an N-Leu-specific enzyme in both kidney and placenta, confirming the suggestion in Paper II about an endopeptidase activity. This pattern, with cleavages N-terminally to Leu-residues, is partly similar to that observed for insulin degradation. In addition, some aminopeptidase-like cleavages were also observed in both extracts.

In conclusion, the degradation of C-peptide in kidney and placenta follows similar patterns, dominated by endopeptidase N-Leu specific cleavages.

6.4 SURFACE PLASMON RESONANCE-BASED STUDIES OF DIFFERENTIAL PROTEIN BINDING TO PROINSULIN C-PEPTIDE (PAPER IV)

Proinsulin C-peptide binding to cell surfaces is shown by surface plasmon resonance-based detection of C-peptide interactions with human serum and detergent solubilized cellular/tissue components. Biotinylated C-peptide and biotinylated scrambled C-peptide were immobilized on streptavidin-coated surfaces of a Biacore sensor chip. The human serum and the detergent solubilized cellular/tissue components showed stronger binding to C-peptide than to scrambled C-peptide. Pre-incubation of cell extracts with free C-peptide lowers these interactions. Additionally, interactions of immobilized C-peptide with extracted membrane components could be blocked by antibodies directed against C-peptide. Molecules bound to the chip were eluted by utilizing the micro-recovery system of the Biacore 3000 instrument. The recovered material was digested with trypsin. The peptides generated were analyzed by MALDI-MS and some were analyzed by direct sequence analysis using tandem MS. Proteins were identified by mass mapping and by searching in databases for the sequences obtained.

In conclusion, the results show the existence of C-peptide interacting molecules in serum and cell/tissue preparations, and demonstrate that this binding can be studied by the SPR technique.

6.5 ENHANCED PROTEIN RECOVERY USING A LARGE-SURFACE BIOSENSOR CHIP (PAPER V)

Sufficient recovery is important in biosensor technology for identification of binding partners and characterization of their molecular and biological properties. A new biosensor instrument based on SPR technology (Cobi R100, Vir Biosensor, Denmark) was tested for affinity purification of anti-C-peptide antibody from a protein mixture and for enhanced protein recovery. Neutravidin was immobilized on plastic chips with a large gold surface (effective area 26 mm^2) followed by coupling of biotinylated human C-peptide. The monoclonal antibody directed against C-peptide was tested for recovery. For elution of the antibody from the C-peptide labeled biosensor chip, 60 µl 5% formic acid was introduced into the flow cell using the two-bubble system and allowed to contact the chip surface for 3 min. Following this treatment, the 60 µl 5% formic acid solution was pumped out and collected for analysis. After SDS/PAGE and silver-staining, the 25 and 50 kDa protein bands (corresponding to the light and heavy antibody chains) were excised and in-gel digested with trypsin followed by MALDI-MS for identification. The capability of the biosensor instrument for affinity purification of proteins was demonstrated (Fig. 6), and the protein recovery was 30-fold greater than demonstrated in other instrumentation.

In conclusion, a large active surface area in combination with the two-bubble approach provides protein purification and enhanced recovery of a bound protein in concentrated form. The amounts are sufficient for multiple structural analyses and functional characterizations.

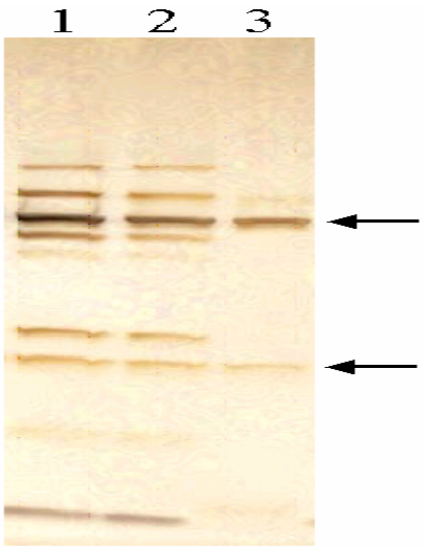

Figure 6. Affinity purification of the anti-C-peptide antibody from a mixture with six other proteins. Lane 1 shows SDS/PAGE of the protein mixture including the antibody; lane 2, the flow-through with much of the antibody removed by attachment to the C-peptide labeled chip; and lane 3, the fraction eluted with the two-bubble system, demonstrating re-purified antibody (with the two chains at the arrows) largely free from the other proteins.

6.6 ELECTRO-IMMOBILIZATION OF A PEPTIDE LIGAND FOR PROTEIN AFFINITY PURIFICATION IN A QUARTZ CRYSTAL MICROBALANCE INSTRUMENT (PAPER VI)

New immobilization techniques and attachment chemistries are important to increase the efficiency of chip-based biosensor technology for affinity purification of proteins. A new biosensor instrument (Attana 80, Attana AB) based on the QCM technology was recently introduced. For this instrument, an electro-immobilization technique was developed where the application of different potentials to the chip surface enables attraction of charged ligands for covalent attachment. The effect was successfully demonstrated using the proinsulin C-peptide as a model for a low-pI highly acidic peptide, normally difficult to localize for covalent attachment to activated surface carboxyl groups. The resulting chip was used in the QCM biosensor instrument to capture anti-C-peptide antibody which was subsequently eluted using the two-bubble system and identified by mass spectrometry.

In conclusion, the method is highly reproducible through repeated cycles and provides affinity purification of proteins at real-time monitoring by electro-immobilization of ligands with a charge that normally prevents efficient coupling to the activated chip surface.

7 CONCLUSIONS

- C-peptide stimulation of phosphorylation of MAPKs is promoted by the presence of three conserved glutamic acid residues in C-peptide, and by helical propensity in the N-terminal segment.
- C-peptide C-terminal pentapeptide is rapidly degraded in serum by an aminopeptidase activity. The full-length C-peptide is differently degraded, apparently by an endopeptidase activity, and a novel N-terminal carbamate modification was detected.
- C-peptide is degraded by an N-Leu-specific enzyme in both kidney and placenta. In addition, some aminopeptidase-like cleavages are observed in both extracts.
- Human serum and detergent-solubilized cell/tissue components show stronger binding to C-peptide than to scrambled C-peptide.
- A large active biosensor chip surface area in combination with the two-bubble approach for elution provides protein purification and enhanced recovery of a bound protein in concentrated form.
- A novel electro-immobilization technique of peptide ligands is demonstrated using biosensor technology based on the quartz crystal microbalance principle for real-time monitoring of affinity purification of proteins.

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“I don’t mind YOU being different, but allow ME to be different from YOU”

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