Ion-Transfer Voltammetric Behavior of Protein Digests at Liquid|Liquid Interfaces

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Ion-Transfer Voltammetric Behavior of Protein Digests at Liquid|Liquid Interfaces

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The development of new methods for the detection of proteins and peptides is of widespread importance. In this work, the electrochemical behavior of peptide mixtures resulting from proteolytic digestion of proteins was investigated at the polarized liquid|liquid interface (or the interface between two immiscible electrolyte solutions, ITIES). The influence of pepsin digestion on three proteins (hemoglobin, lysozyme, and cytochrome *c*) was studied, and it was revealed that resulting cyclic voltammograms of the three protein digests were different due to the unique peptide mixtures for a given protein. Differential pulse stripping voltammetry of protein digests enabled the detection of digested proteins at concentrations ranging between 0.55 and 4.22 μM. A limit of detection of 0.55 μM of the initial concentration of protein was achieved, demonstrating the analytical possibilities of such an electrochemical method. These results show that ion-transfer voltammetry offers the opportunity to study and develop label-free detection of peptides resulting from enzymatic digestions of proteins and may thus have a role in development of new proteomic technologies.

The ability to detect and identify proteins has enormous importance in modern science and technology, with potential impacts on numerous areas, including the study and treatment of diseases, food, and environmental science. Although there is a strong reliance on two-dimensional gel electrophoresis and mass spectrometry methods[1−6] in modern protein science, these methods are not without their problems.[7,8] New methods for sample handling and preparation, protein detection, covalent modification analysis, and biomarker discovery are needed in order to accelerate development of protein technology. Electrochemistry offers fruitful scope for investigations that can solve some of these problems. For example, detection and quantitation of proteins has attracted an increasing amount of interest for biomedical and diagnostics applications, for which electrochemistry offers sensitive and label-free options.[9]

Although electrochemistry at the liquid|liquid interface has been studied intensively for a number of decades, interest in its analytical capabilities has increased significantly recently.[10−12] This form of electrochemistry, also referred to as electrochemistry at the interface between two immiscible electrolyte solutions (ITIES), has the advantage that it is based on the transfer of ions from one electrolyte phase to the other. Therefore, the electrochemical behavior of species which are not readily oxidized or reduced can be investigated and harnessed. Furthermore, miniaturization and portability are easily achievable as for other electrochemical approaches, increasing the possibility of operation outside of the laboratory. The electrochemical behavior of proteins at the ITIES has growing interest and is rather complex,[13−20] involving protein adsorption at the interface and ion transfer across the interface, not necessarily protein transfer.[16−19] However, oligopeptide transfer across the polarized interface has demonstrated promising electroanalytical capabilities,[21−23] which were improved by miniaturization of the interface.[21,24] The miniaturized ITIES, or μITIES, which offers enhanced analytical performances, may be prepared by a variety of approaches such as localization of the interfaces within the pores of microporous membranes

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prepared by laser ablation of polymer membranes[25−29] or chemical etching of silicon membranes,[30] or by creation of microscopic arrays of oil droplets on a solid electrode substrate.[31] Electrochemistry at these microscopic interface arrays has been used for the detection of urea,[26] creatinine,[27] nitrate,[29] adenosine phosphate,[28] dopamine,[32] β-blockers,[24] and oligopeptides.[21] Stripping voltammetry (SV) can also be used to improve electroanalytical performance, in which case a preconcentration step is implemented by constant-potential transfer of the analyte ions from the aqueous (sample) phase into the organic phase. In the subsequent detection step, a voltammetric scan is applied to back-extract the analyte into the aqueous phase. Submicromolar concentrations of oligopeptides[21] and the β-blocker propranolol[24] were determined using SV at arrays of μITIES localized within silicon membranes.

We present here investigations into the electroanalytical behavior at the ITIES of peptide mixtures produced by enzymatic digestion of proteins. Three proteins, hemoglobin (Hb), lysozyme (Lz) and cytochrome *c* (Cyt *c*), were digested by the proteolytic enzyme pepsin. The resulting peptide mixtures were analyzed by electrochemistry at the ITIES. Electroanalytical possibilities were further investigated by SV of Hb digested by either pepsin or trypsin at a μITIES array. Pepsin and trypsin are proteases which are frequently used in protein studies.[33] They both cleave peptide bonds but at different locations. Proteolytic digestion results in a mixture of peptides, which is characteristic for a particular protein. This mixture is usually characterized by combinations of mass spectrometry, gel electrophoresis, high-performance liquid chromatography, or capillary electrophoresis.[1−6,34] From an electrochemical point of view, there have been some reports of the use of ion-selective electrodes (ISEs) to study protamine digestion by trypsin.[35−38] In this paper, we report studies into the electroanalytical behavior of enzymatically digested proteins based on ion-transfer voltammetry at the ITIES, as a step toward development of new methods for protein identification. This is conceptually illustrated in Figure 1.

EXPERIMENTAL SECTION

Reagents. All reagents were purchased from Sigma-Aldrich Ireland Ltd. and used as received, with the exception of 1,6-dichlorohexane (1,6-DCH) which was purified following the published procedure.[39] The organic phase electrolyte salt was prepared by metathesis of bis(triphenylphosphoranylidene) ammonium chloride ($BTPPA^+Cl^-$) and of potassium tetrakis(4-chlorophenyl) borate (K^+TPBCl^-) to obtain $BTPPA^+TPBCl^-$ following the published procedure.[25] For studies involving liquid organic phase, this was prepared in 1,2-dichloroethane (1,2-DCE). For gelled organic phases, the organogel phase was prepared using purified 1,6-DCH and low molecular weight poly(vinyl chloride) (PVC).[21]

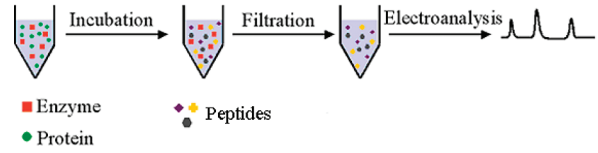

Figure 1. Experimental strategy for electrochemical protein discrimination.

Scheme 1

Cell 1

$Ag_{(s)}$ | $AgCl_{(s)}$	x µM protein digest (or protein) in 10 mM HCl (aqueous)	10 mM $BTPPA^+$ $TPBCl^-$ in 1,2-DCE	10 mM $BTPPA^+$ Cl^- in 10 mM LiCl in H_2O	$AgCl_{(s)}$ | $Ag_{(s)}$

Cell 2

$Ag_{(s)}$ | $AgCl_{(s)}$	x µM protein digest (or protein) in 10 mM HCl	10 mM $BTPPA^+$ $TPBCl^-$ in 1,6-DCH organogel	10 mM $BTPPA^+$ Cl^- in 10 mM LiCl in H_2O	$AgCl_{(s)}$ | $Ag_{(s)}$

Electrochemical Details. Experiments were carried out at both a large ITIES and at an array of μITIES. Voltammetry at the ITIES was performed with a CH Instruments 660B potentiostat (IJ Cambria, Burry Port, United Kingdom). The ITIES cell is composed by two Ag|AgCl electrodes (one in each phase) and two Pt mesh counter electrodes (one in each phase). A customized glass liquid|liquid electrochemical cell was used, fabricated by AGB Scientific Limited (Dublin, Ireland). The geometric area of the ITIES in this cell was 0.785 cm^2, and it was flat in appearance. The composition of the cell is given in Scheme 1, cell 1. Cyclic voltammetry (CV) experiments were used for the electrochemical characterization of the ion-transfer processes of the protein digests.

Voltammetry at the μITIES array was performed with an Autolab PGSTAT30 potentiostat (Ecochemie, The Netherlands). Differential pulse stripping voltammetry (DPSV) was used for studies at the μITIES array, with the following parameters: pulse amplitude, 25 mV; pulse time, 0.05 s. Microporous silicon membranes were used to define the μITIES array. The micropore arrays were fabricated from 525 μm thick silicon wafers using a combination of dry and wet silicon etching to thin the wafers and etch pores through the thinned portions.[30] The micropore array consisted of eight micropores in a hexagonal close-packed arrangement. Each individual pore was 52 μm in diameter and the pore-to-pore (center-to-center) distance was 500 μm. This membrane was sealed to a glass tube (6 mm external diameter, 3 mm internal diameter) using silicone rubber (RS Components, stock number 555-588). The organogel phase was then placed inside the glass cylinder, as described previously.[21,24] The composition of the cell is shown in Scheme 1, cell 2.

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Protein Digestion. Protein digestions were carried out according to published procedures.[34,40] For peptic digest preparation, the analyte protein and pepsin (from porcine gastric mucosa, 2500−3500 units mg^{-1}) were dissolved together in 1.5 mL of 10 mM HCl at an enzyme-to-protein molar ratio of 1:25. This mixture was incubated at 37 °C for 2 h. To prepare tryptic digests, Hb was added to a solution of 0.5 g L^{-1} trypsin (at an enzyme-to-protein molar ratio of 1:25) and 0.2 g L^{-1} of ethylenediaminetetraacetic acid (pH 7.0−7.6). This mixture was incubated at 37 °C for 16 h. Following the indicated reaction time, for both digest types, the protein digest was then separated from undigested material by centrifugation using Ultrafree-MC centrifugal filter devices from Millipore (purchased from Sigma-Aldrich Ireland Ltd.). Separation conditions were 4500 rcf for 40 min for the peptic digest and 15 700 rcf for 40 min for the tryptic digest. Samples were stored at +4 °C prior to analysis and were equilibrated at room temperature prior to electrochemical analysis. Analysis involved additions of aliquots of the protein digest to the aqueous phase of the electrochemical cell using a micropipet.

RESULTS AND DISCUSSION

Influence of Protein Digestion on Electrochemistry at the ITIES. Figure 2A shows the impact of enzymatic digestion on the electrochemical behavior of Hb at the ITIES. The dashed curve is typical of the electrochemical behavior of Hb at the ITIES,[17] whereas the solid curve corresponds to the electrochemical signal of digested Hb. On the forward scan, when a broad peak at +0.72 V was observed for Hb, a much sharper peak was detected at +0.80 V for the Hb digest. On the reverse scans, three peaks or waves were recorded for the Hb digest, at +0.61, +0.68, and +0.73 V, whereas a single broad peak was observed for the undigested Hb. These results demonstrate the influence of enzymatic digestion on protein electrochemistry at the ITIES and show that single-peak CV responses of a protein are transformed to multipeak CVs of the peptide mixture resulting from enzymatic digestion.

In addition to the protein digestion CVs, a series of control experiments demonstrated that the electrochemical signal obtained upon addition of the digested protein solution to the aqueous phase of the electrochemical cell resulted from the products of the enzymatic digestion and was not an artifact associated with the experimental materials. The results of these control experiments are shown in Figure 2B. In these cases, three control solutions, comprising (i) 10 mM HCl, (ii) pepsin in 10 mM HCl, and (iii) Hb in 10 mM HCl, were incubated and filtered as for the protein digestion experiments. In all three cases, no ion transfer was recorded within the available potential window. These results prove that the data presented in Figure 2A are due to the enzymatic digestion of Hb and not to the transfer of pepsin, Hb, or impurities from the filter. Similar results to those shown in Figure 2A for Hb were also obtained for Cyt *c* and Lz, indicating that peptides from digested proteins can be electrochemically detected at the ITIES.

Comparative Electrochemistry of Digested Proteins at the ITIES. The transition of the electrochemical behavior from predigestion to postdigestion is indicative of changes in the ion-transfer processes occurring at the ITIES, either the nature of the ion transfer or the identity of the transferring ions. Sawada and Osakai demonstrated that the transfer potential of oligopeptides at the ITIES is dependent on their hydrophobicity.[23] According to the ExPaSy server,[41] peptic digestion of Hb, Lz, and Cyt *c* results in 166, 17, and 13 peptide fragments, respectively. These are mainly positively charged peptides, due to the acidic nature of the digestion conditions, which vary in size and hydrophobicity. Amino acids and small peptides need the presence of an ionophore in the organic phase to assist their transfer across the ITIES and enable their detection.[21−23,42] Dibenzo-18-crown-6-ether is one such ionophore used, but in this work, it was found that its addition to the organic phase did not facilitate the transfer of the protein digests (not shown). This means that the electrochemical signals shown in Figure 3 are due to so-called unfacilitated (or simple) ion-transfer reactions, which occur for ions that possess sufficient inherent hydrophobicity to transfer across the ITIES within the available potential window. Effectively, the largest and the most hydrophobic peptide fragments of Hb, Lz, and Cyt

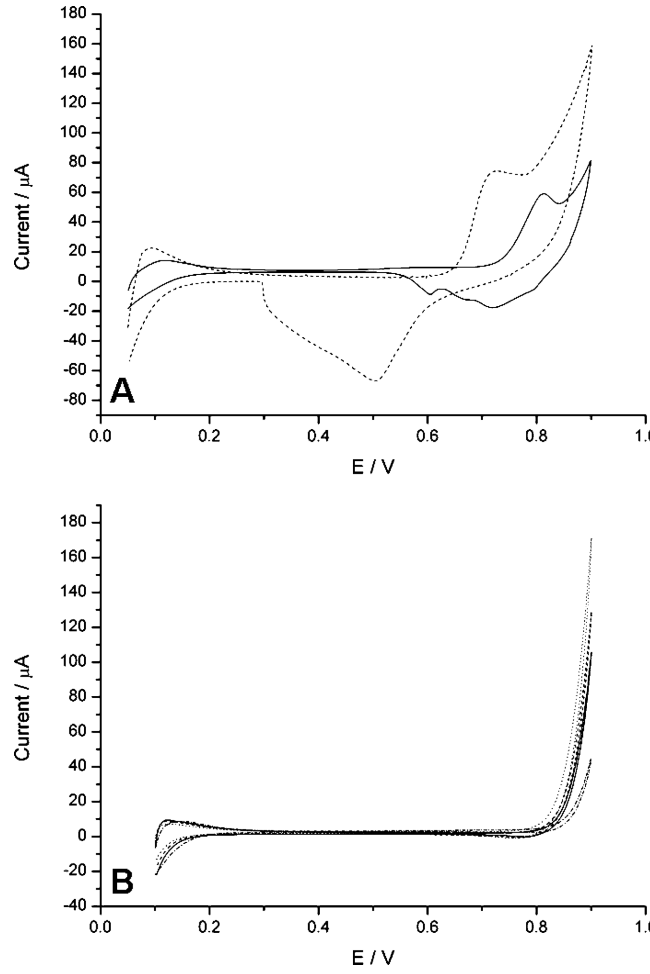

Figure 2. (A) CV of 2.7 μM of Hb (---) and of 23.1 μM of Hb peptic digest (−) in the aqueous phase. (B) CV of control experiments. Blank (−), HCl incubated and filtered (-··-), pepsin incubated and filtered (---), Hb incubated and filtered (···). ν = 5 mV s^{-1}. Electrochemical cell 1.

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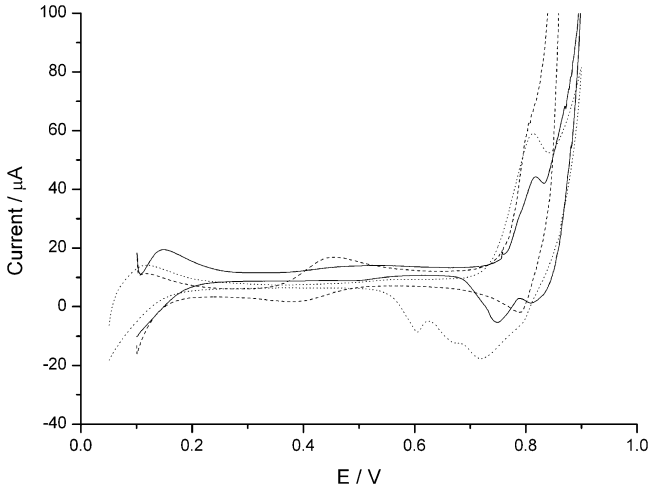

Figure 3. CV after peptic digestion. CVs of 52.6 μM of lysozyme digest (−), of 31.2 μM of Cyt *c* digest (---), and of 23.1 μM of Hb digest (•••) in the aqueous phase. ν = 5 mV s^{-1}. Electrochemical cell 1.

Table 1. Electrochemical Characteristics of Protein Digests Studied

protein	protein concn/μM	protein/enzyme ratio (mol/mol)	E_f/V[a]	Q_f/mC[b]	E_r/V[c]	Q_r/mC[d]
Hb	370	12:1			+0.61	
			+0.72	1.34	+0.68	−0.39
					+0.73	
Lz	270	7:1	+0.82	1.03	+0.74	−0.09
Cyt*c*	160	6:1	+0.81	1.48	+0.78	−0.15

[a] E_f: forward peak potential. [b] Q_f: forward peak charge. [c] E_r: reverse peak potential. [d] Q_r: reverse peak charge.

c are detected by the CVs shown in Figure 3 because these peptides transfer across the ITIES in the absence of an organic phase ionophore. The CV signatures of the individual protein digests are different because they have been digested into different peptide fragment mixtures. Table 1 summarizes the protein/pepsin mixture conditions and the electrochemical characteristics of the three protein digests studied. Hb has many fragments compared to the other two proteins studied, and its CV exhibits three peaks on the reverse sweep. Conversely, the other two proteins provide lower fragment numbers, and their CV signatures produce one peak on the reverse half-cycle. Thus, there is a qualitative agreement between the number of peptide fragments and the detectable electrochemical processes obtained with a simple technique such as CV.

Comparison of Peptic and Tryptic Digests at the μITIES Arrays. The detection of Hb fragments, digested by either pepsin or trypsin, was investigated by DPSV at μITIES arrays in which the organic phase was gelled. This method was previously applied to the detection of oligopeptides[21] and drugs.[24] It consists of two steps, a preconcentration step and a detection step. This approach was employed here because of the greater numbers of peaks detected on the reverse sweeps of the CVs of digested Hb, thus offering greater identification capability, as well as the better low-concentration detection characteristics of DPSV. For these experiments, an array of eight micropores to define the μITIES array was used, so as to achieve radial diffusion to each micropore.[43]

Figure 4 shows the voltammograms for DPSV of Hb (Figure 4A), of Hb digested by pepsin (Figure 4B), and of Hb digested by trypsin (Figure 4C). The presence of Hb in the aqueous phase resulted in the appearance of a broad shoulder, which increased with the concentration of Hb in the aqueous phase (Figure 4A). When the Hb peptic digest was present in the aqueous phase, two shoulders (around +0.8 and +0.95 V) were observed (Figure 4B). Increasing concentrations of Hb digests caused an increased transfer current at these peak potentials. Upon addition of the tryptic protein digest to the aqueous phase, a peak was observed at +0.75 V, the magnitude of which increased with the concentration of digest added to the aqueous phase (Figure 4C). These results show major differences in the electrochemical behavior of Hb, Hb peptic digest, and Hb tryptic digest.

Although the data of Figure 4A−C show some discrimination between Hb and its peptic and tryptic digests, it should be noted that voltammogram for Hb at the aqueous phase|organic gel interface was not as clear as was achieved at the ITIES using an organic liquid phase.[17,20] The poor response for Hb at the gelled μITIES array is probably due to the mechanism of the electrochemical behavior of proteins at the liquid|liquid interface. Previous studies have demonstrated that proteins adsorb at the ITIES and then facilitate transfer of organic anions across the interface.[17−20] The electrochemical signal was observed when the pH of the aqueous phase was lower than the pI of the protein.[17−19] At pH < pI, proteins are positively charged and facilitate the transfer of the organic electrolyte anion. The nature of the organic electrolyte therefore plays an important role in electrochemical signal generation, unlike the nature of the cation of the organic electrolyte.[20] Proteins have not been shown to transfer across the ITIES unless a surfactant was present in the organic phase to facilitate transfer.[14−16] In the present study, protein adsorption at the gelled ITIES and its facilitation of anion transfer probably does not occur to the same extent as at the pure (ungellified) ITIES. This may be a consequence of slow diffusion of the organic phase electrolyte anion in the organogel phase within the micropores, giving rise to a lower electrochemical sensitivity.

The difference between the voltammograms for the peptic and the tryptic digests of Hb is due to the different peptide mixtures produced by these digestions. Although peptic digestion leads to a greater number of peptides,[41] the voltammogram of the Hb peptic digest did not necessarily lead to a greater number of voltammetric peaks. This may be because the peptides produced are smaller and hence less hydrophobic and may transfer outside of the available potential window. Two different data analysis approaches were taken to try to improve the signal resolution of the voltammograms resulting from DPSV of the digested protein. The first was background subtraction, i.e., subtraction of the DPSV signal obtained when [Hb] = 0 μM from that obtained when [Hb] ≠ 0, (Figure 4D−F). Subtraction of the background did not improve the peak shape for the Hb voltammograms (Figure 4D). However, better peak resolution was observed for both the peptic

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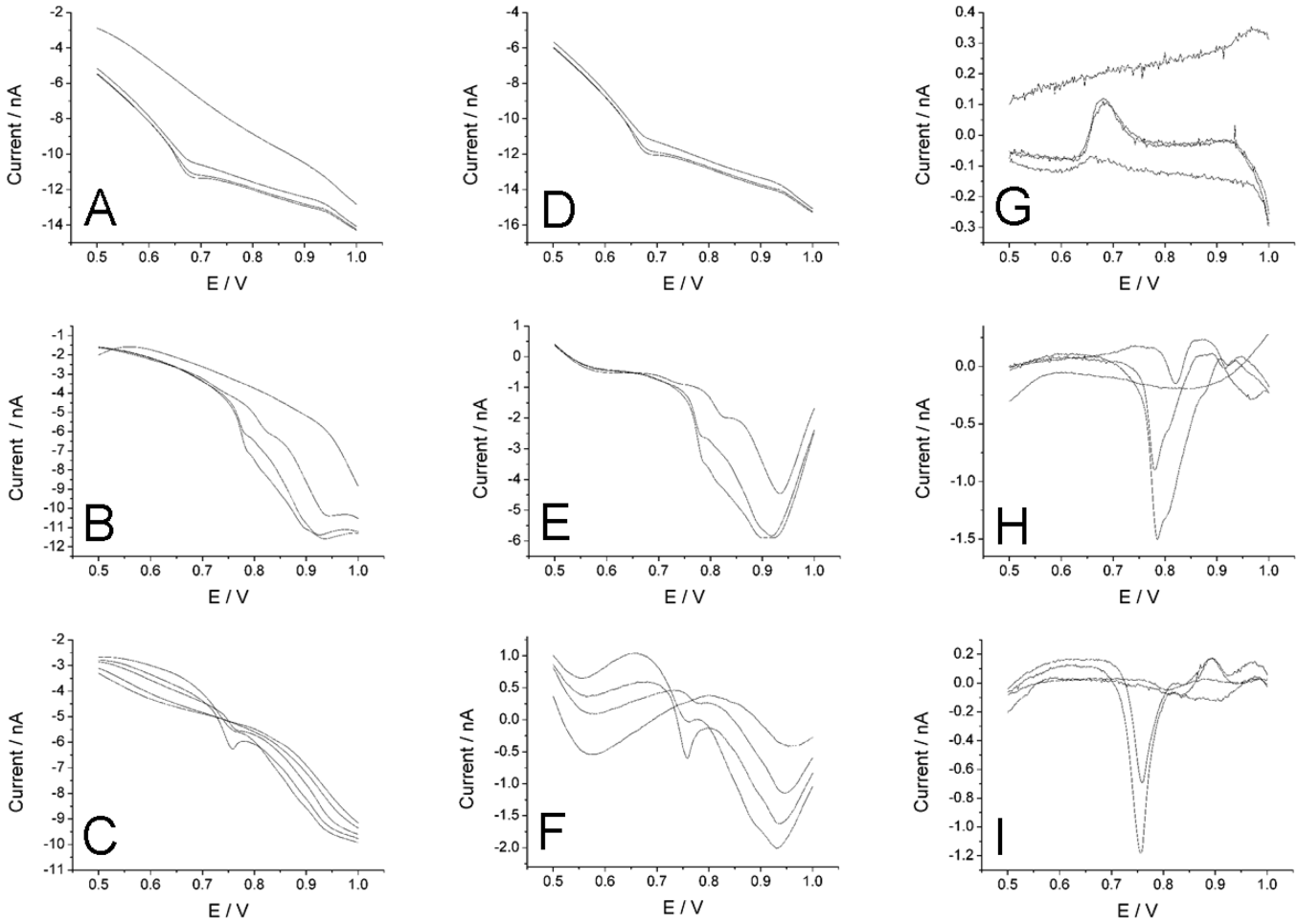

Figure 4. DPSV of (A) Hb, (B) Hb peptic digest, (C) Hb tryptic digest; DPSV with background subtraction of (D) Hb, (E) Hb peptic digest, (F) Hb tryptic digest; DPSSV with subtraction of a 0 s preconcentration scan of (G) Hb, (H) Hb peptic digest, (I) Hb tryptic digest. Concentrations from top to bottom are, for panels A and G, 0, 1.96, 3.85, 5.06 μM; for panels B, C, H, and I, 0, 1.63, 3.21, 4.22 μM; for panel D, 1.96, 3.85, 5.06 μM; for panels E and F, 1.63, 3.21, 4.22 μM. Concentrations are for electrochemical cell 2.

and the tryptic digests of Hb (Figure 4E, parts and F). For the Hb peptic digest, a peak at +0.9 V was clearly observed together with a smaller peak at ca. +0.8 V (Figure 4E). The peak at +0.9 V increased with the concentration before reaching saturation. For the smaller peak, the peak potential shifted to less positive values when the concentration increased. For Hb tryptic digest, two well-resolved peaks, at +0.93 and +0.75 V, were observed (Figure 4F).

The second data processing method was the subtraction of the signal recorded when no preconcentration step was applied (Figure 4G−I). This method at the liquid|liquid interface, differential pulse subtractive stripping voltammetry (DPSSV), has been described previously.[21] Increasing Hb concentration in the aqueous phase did not lead to an increase of the electrochemical signal (Figure 4G) with this approach. DPSSV tended to produce a sharper peak than DPSV with regular background subtraction. A single peak, around +0.78 V, was observed for the peptic digest of Hb (Figure 4H) with a slight shoulder appearing above a concentration of 3.21 μM. Similarly, a sharp peak at +0.75 V was observed for the Hb tryptic digest (Figure 4I).

Effect of DPSV Preconcentration Time. The preconcentration step is an important element of the DPSV method, as it offers the opportunity for substantial improvements in detection limits and sensitivity. The influence of preconcentration time on the voltammograms is shown when no Hb or Hb digest is present (Figure 5A), when [Hb] = 3.85 μM (Figure 5B), when [Hb_{peptic}] = 3.21 μM (Figure 5C), and when [$Hb_{tryptic}$] = 3.21 μM (Figure 5D). During the preconcentration step, the potential applied should drive the transfer of species across the aqueous|organogel interface. For Hb, there was no difference between the voltammogram after 0 s of preconcentration and after 60 s of preconcentration. When Hb digest was present in the aqueous phase, the voltammograms differed when the preconcentration time varied (Figure 5, parts C and D). A peak appeared at ca. +0.75 V after a 60 s preconcentration time, which indicates that peptides resulting from the enzymatic digestion transfer across the aqueous|organogel interface. However, no difference is observed, in both cases, around +0.9 V, suggesting that the signal recorded at this potential is not due to the transfer of peptides. It might be due to interactions of larger peptide residues that adsorb at the interface and interact with ions of the organogel in a similar manner to Hb.[17−20]

Concentration Dependence. Calibration curves were constructed for the results from DPSV with background subtraction and from DPSSV. The Hb peptic digest peak at +0.9 V increased linearly with concentration before reaching saturation at 2.69 μM, perhaps due to adsorption of peptides at the interface. The peak currents of the two peaks for the Hb tryptic digest increased

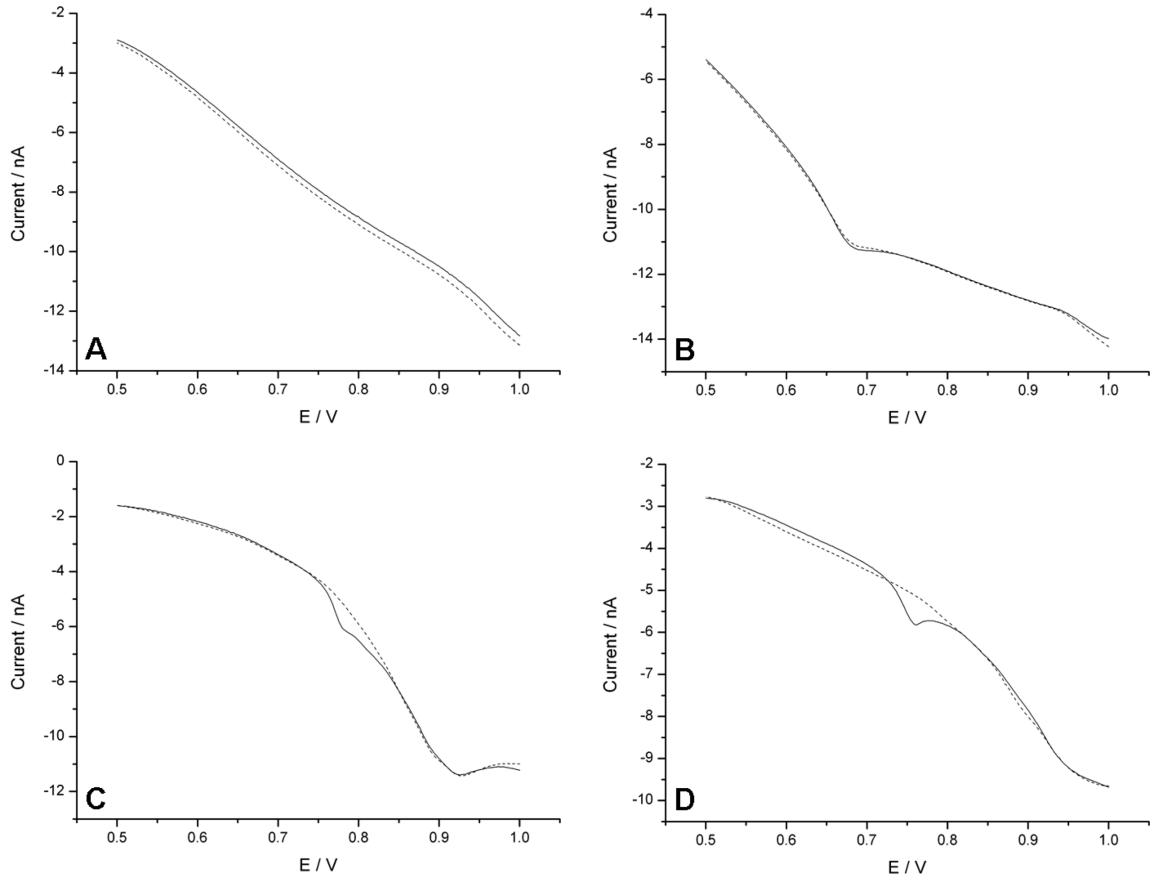

Figure 5. DPSV with 0 s (– – –) and 60 s (—) preconcentration at (A) blank aqueous solution, (B) 3.85 μM Hb concentration, (C) 3.21 μM Hb peptic digest, and (D) 3.21 μM tryptic digest; electrochemical cell 2.

linearly with concentration without reaching saturation. However, the peak at +0.95 V was present at a concentration of 0.55 μM, whereas the peak at +0.75 V only appeared when the concentration was 2.16 μM. The linear best-fit equations for the current–concentration calibration curves were $y = -0.42x + 0.81$ ($R = 0.992$, $N = 5$) for the peak at +0.75 V and $y = -0.49x - 0.53$ ($R = 0.999$, $N = 8$) for the peak at +0.95 V. The slopes (sensitivities) of these two calibration lines were similar, indicating that the charge and diffusion coefficient of the species involved were similar. The limit of detection for the Hb tryptic digest using the peak at +0.9 V was calculated to be 0.55 μM.[44] Calibration curves for both Hb digests were also plotted using the peak current values from the DPSSV data analysis. The straight-line equations for these calibration curves were $y = -0.55x + 0.90$ ($R = 0.992$, $N = 6$) for the Hb tryptic digest peak at +0.75 V and $y = -0.48x + 0.49$ ($R = 0.997$, $N = 7$) for the Hb peptic digest peak at +0.78 V. The high value for the intercept prevented calculation of the limit of detection for these data.

General Discussion. The major detection platform in proteome analysis is mass spectrometry (MS), which allows the identification and the characterization of many proteins of biological interest. The complexity of the samples studied, however, requires the use of preparative steps, which can be performed by either liquid chromatography (LC) or gel electrophoresis. For some samples, a preparative step before gel electrophoresis may even be required.[45] Thus, the established methods of proteomics are multidimensional analytical systems, involving two or three sample preparation dimensions prior to MS detection. Other alternative sample preparative methods are also being investigated such as microwave-assisted methods.[46] With electrospray ionization MS, protein concentrations are usually detectable in the submicromolar and micromolar ranges,[47,48] which is similar to the level of detection achieved with SV at the μITIES. Matrix-assisted laser desorption/ionization (MALDI) MS is a much more sensitive technique which enables, under optimal conditions, the detection of picomolar concentrations of peptides,[49] which is beyond the limit of detection of SV at the μITIES. However, all of these methods employ high-powered sample preparation and separation methods in conjunction with the detection, unlike the simple approach based on voltammetry at the ITIES presented here.

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CONCLUSIONS

The results of a study of the influence of protein digestion on electroanalytical behavior at ITIES and μITIES arrays have been presented in this paper. These results demonstrate that enzymatic digests of proteins are electroactive at these liquid|liquid interfaces and that the electrochemical behavior of the peptide mixtures produced by enzymatic digestions is different from that of the undigested proteins. The enzymatic digestion enables an electrochemical signature of the protein to be obtained, which is not dependent on redox characteristics of the fragments but on their ability to transfer across the ITIES. This means that the method is label-free and depends only on the ability of ions to transfer across the interface. These studies have also shown that the electrochemical behavior depends on the enzyme used for the proteolysis. Although it will not compete with present established methods for protein characterization and detection, based on the results presented, the approach of enzymatic digestion combined with detection by electrochemistry at the ITIES may be useful as a simple characterization method in protein chemistry and protein technology. This offers extreme simplicity in terms of instrumentation but, at present, does not offer the sensitivity of widely used methods, such as those based on combinations of multidimensional separations and MS detection. Clearly, combination of such sample preparation and separation methods with voltammetry at the ITIES offers scope for improved electrochemical detection performances.

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