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Short Communication

Discrimination of bovine and porcine insulin by higher-order derivative UV-spectroscopy

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Introduction

Insulin is a peptide hormone, whose structure and function have been investigated extensively due to its biological importance and as a model system for protein chemistry. It contains 51 amino acid residues which are arranged in two polypeptide chains, A (21 residues) and B (30 residues). The A-chain is linked to the B-chain by two disulphide bridges (A7–B7 and A20–B19); an additional one between A6 and A11 helps stabilize the folded form of the molecule.

The aromatic amino acids comprise four tyrosine residues (A14, A19, B16, B26) and three phenylalanine residues (B1, B24, B25). They are important moieties of insulin not only because they are the chromophoric groups, but also because they play an important role in aggregation phenomena [1, 2] and are among the invariant residues which are suspected to be responsible for receptor–hormone interaction [3].

Although the active form is a monomer, the insulin molecule in solution tends to form dimers, and in the presence of zinc, three dimers combine to form hexamers, and possibly higher oligomers, in a way which changes the environment of the chromophores. For the examination of solution structure of various forms of the molecule, UV-spectroscopy [4, 5] and circular dichroism (CD) [6–8] have been used extensively.

Although UV–vis spectroscopy is used frequently, the near UV-absorption of proteins lacks detailed fine structure, due to the overlapping bands caused by the complex environment surrounding the chromophores, where UV-difference spectroscopy has proved to be helpful [4]. On the other hand, CD spectroscopy has been applied by several workers for studying the conformation and association properties of insulins; in these studies the contribution of aromatic residues to the CD spectrum were deduced by analogy with the corresponding near UV-absorption spectra, but it has been stated that tyrosyl and phenylalanyl contributions could only be resolved approximately [7, 8].

Additionally, the tendency of insulin towards aggregation and desamidation [9] may give rise to turbidity in pharmaceutical preparations which creates a problem during storage and also limits the application of the aforementioned techniques.

Derivative spectroscopy is now recognized as a tool for the analysis of drug substances, especially in cases where spectral interferences obscure the desired spectral information [10, 11]. Second- and fourth-order derivatives have been applied to the quantitation and identification of amino acid residues by several workers [12–15]. The method has also been utilized for the examination of micro environments [16] and states [17–18] of phenylalanine, solvent accessibility of tyrosine [19] and for examining the conformational changes [20] and interactions [21] of various proteins.

Insulins obtained from different sources show differences in their amino acid sequences

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which may alter the structure, association, biological activity and stability of the molecule. Beef and pork insulins which differ by eight to 10 residues in the A chain (pork: thr.ser.ile; beef: ala.ser.val) have long been used in diabetes therapy and have served as model compounds for studying insulin chemistry. UV-derivative spectroscopy has not been used extensively for the comparison of insulins or for the determination of their degradation states. In this study, the use of higher even-order derivatives has been investigated for a comparative study of beef and pork insulins. The applicability of this technique has been preliminarily examined to study the changes in insulin structure during oxidative degradation.

Experimental

N-acetyl phenylalanine ethylester, *N*-acetyl tyrosinamide, beef and pork insulin (24 IU mg^{-1}, 0.5% Zn content) were obtained from Sigma Chem. Co. (Poole, Dorset, UK). Solutions of amino acids and proteins were prepared at appropriate concentrations in 0.06 M phosphate buffer (pH 7.4) and 0.1 M NaOH (pH 12.85). Spectra were recorded with a Perkin–Elmer Lambda 5 spectrophotometer (Beaconsfield, UK), transmitted to a PE data station and stored on disk for further processing. A standard set of scanning conditions was employed in order to facilitate comparisons between spectra: bandwidth, 2 nm; response, 0.5 s; derivative computation interval, 1.0 nm; range, 230–300 nm and 230–340 nm. Derivative spectra were generated on the Data Station with a simple DERIV.OY program supplied by Perkin–Elmer operating on the principle of digital arithmetic calculation by the convolution method. Even-order derivatives up to eighth order were generated for all spectra previously recorded and stored on disk. A standard set of processing parameters was adopted (1-nm data point interval, 13-point convolution interval). The amplitude scaling factors for the higher order derivatives were determined empirically.

Stress experiments on insulin degradation were conducted on solutions of pork and beef insulins prepared in pH 7.4 phosphate buffered saline (0.1 M) placed in rubber-capped multi-dose vials fitted with inlet and outlet needles. Air bubbles were drawn through the solution by negative pressure with the aid of a water-filled 25-l aspirator bottle connected to the outlet needle, at ambient room temperature. The spectra of these solutions were recorded at the intervals indicated in Fig. 3 on a Shimadzu 160A UV–vis spectrometer (Ankara, Turkey) under the following scanning conditions: slit-width, 2 nm; scan speed, 1500 nm min^{-1}; response, 0.2 s; scan range, 230–300 nm.

Results and Discussion

Typical derivative spectra of insulin are shown in Figs 1 and 2. The peak positions of characteristic absorption bands in the zeroth, second to eighth order derivative spectra of *N*-acetyl tyrosinamide ($AcTyrNH_2$), of *N*-acetyl phenylalanine ethylester (AcPhEt), and of pork and beef insulins are tabulated in Table 1 for solutions at pH 7.4. As can be seen from the data presented, the characteristic bands attributed to aromatic amino acids can be detected in the derivative spectra of insulin. Those associated with tyrosine exhibit shifts to longer wavelengths, varying from 1 to 2 nm, attributable to the change in the chemical environment of this amino acid in the protein. It would be expected that any shift introduced in the position of the derivative peaks by the nature of the derivative algorithm itself should be comparable for both the ester and the amino acid in the protein. Due to the appearance of multiple satellite bands the degree of complexity increases as the derivative order is increased. The computational noise also increases, a feature of all derivative methods employing simple digital filters. It is also notable that the spectral region below 270 nm, where phenylalanine absorbs, shows increasingly more fine structure with increasing derivative order, indicating the possibility of further resolution of these features. Here a shift of 1 nm is observed.

In alkaline solution, the red shift from 275 to 292 nm due to the ionization of *n*-acetyl-tyrosineamide is also observed in both insulin spectra (Table 2 and Fig. 2) along with perturbation of the spectral profile. However, these bands are very broad and not readily useful for characterization purposes. In an attempt to quantify the spectral dissimilarities of pork and beef insulins, the discrepancies between the same order derivative spectra of the insulins were assessed by the root mean squares (RMS) of their differences in buffer pH 7.4 and in alkaline solution, according to the formula:

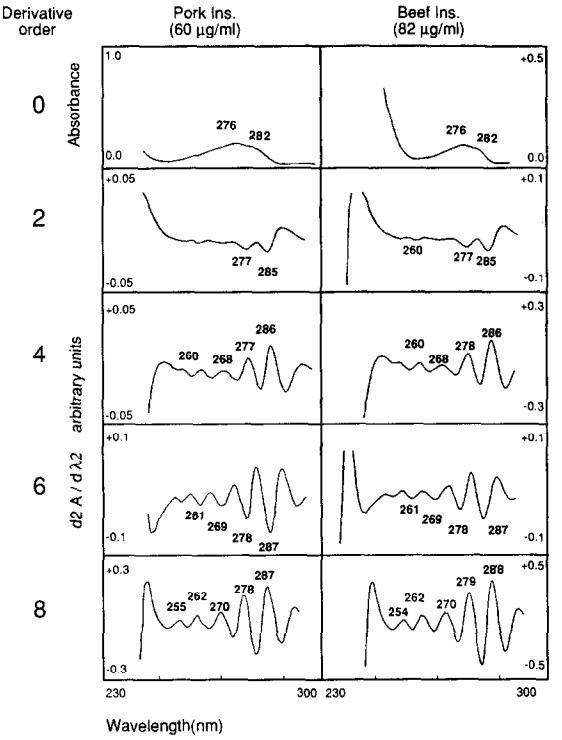

Figure 1
Typical zeroth-order and higher-derivative spectra of beef and pork insulins at pH 7.4, using a Perkin–Elmer Lambda 5 UV-spectrophotometer: slit-width, 2 nm; derivative computational bandwidth, 1 nm.

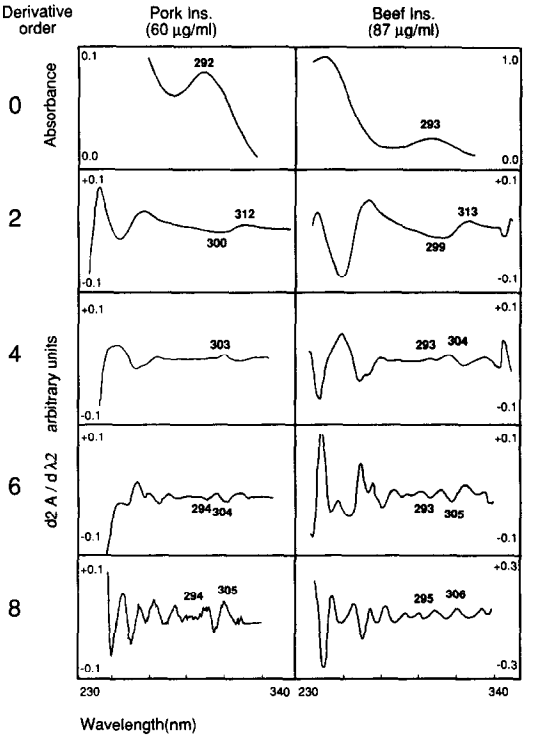

Figure 2
Typical zeroth-order and higher-derivative spectra of beef and pork insulins at pH 12.85. For instrumentation, see Fig. 1.

Table 1
Positions of principal bands observed at pH 7.4 for pork insulin, beef insulin, *N*-acetyl phenylalanine ethyl ester (AcPhEtester) and *N*-acetyl tyrosineamide (AcTyrNH2), using the Perkin–Elmer Lambda 5 UV-spectrophotometer. Slit-width, 2 nm; derivative computational interval, 1 nm

Derivative order	AcPhEt ester	Pork insulin	Beef insulin	$AcTyrNH_2$
zero		283sh	282sh	282sh
		276	276	275
	263s			
	257max			
	252max			
	246sh			
2		285	285	283
		277	277	275
minima	265			
	258		260	
	252			
4		286	286	283
		277	278	276
maxima	267	268	268	
	259	260	260	
	252			
6		287	287	285
		278	278	276
minima	268	269	269	
	259	261	261	
	252		254	
8		287	288	285
		278	279	277
maxima	(noisy)	270	270	
	261	262	262	
	253	255	254	

sh = shoulder, max = maximum.

Table 2
Positions of principal bands observed at pH 12.85 for pork and beef insulin and *N*-acetyl tyrosinamide ($AcTyrNH_2$), using the Perkin–Elmer Lambda 5 UV-spectrophotometer. Slit-width, 2 nm; derivative computational interval, 1 nm. See Table 1 for key

Derivative order	$AcTyrNH_2$	Pork insulin	Beef insulin
0	292	292	293
2	298	300	299
minima	311	312	313
4	293		293
maxima	302	303	304
6	293	294	393
minima	303	304	305
	310		
8	294	300	295
maxima	304	305	306
	311		

$$\mathrm{RMS} = \sqrt{[\Sigma(A_i^{beef} - A_i^{pork})/N]} \quad (1)$$

where $(A_i^{beef} - A_i^{pork})$ is the difference in absorbance of derivative amplitudes for spectra of beef and pork, at wavelength i summed over the N data points.

The statistical significance of these RMS differences was tested by the F-ratio (variance ratio) between the RMS and a 'noise value' calculated from two successive runs of the appropriate insulin spectrum

$$F = \mathrm{RMS}^2_{spectra}/\mathrm{RMS}^2_{noise}. \quad (2)$$

It is probable that the information in the derivative spectra above fourth order are of limited value due to the nature of the complex overlapping satellite bands thereby generated, and the increased noise level [11].

The method was applied to pairs of spectra recorded at pH 7.4 and pH 12.85 over the wavelength range 240–320 nm. As can be seen in Table 3, and the F-ratios (at ∞ degrees of freedom and 0.05 significance level) were found to exceed the tabulated value 1.00 [22], which shows that insulins from the two sources have significantly different spectra under the scanning conditions adopted in this work. Thus it is suggested that this chemometric method may be of value in distinguishing different insulins from each other, even if they contain similar numbers of aromatic amino acids, as in the present case.

Spectra of air-stressed beef and pork solutions (Fig. 3) show similar, but interesting features. The second derivative spectrum of the fresh solution exhibits five well resolved minima at 285 and 279 nm for tyrosine, and at 266, 259 and 252 nm for Phe. After 48 h air stressing, in the zero-order spectra an absorbance increase at 276 nm is observed along with band narrowing. This well known behaviour of tyrosine-containing proteins has been attributed to changes occurring in the conformation of Tyr by various effects, such as solvent perturbation, temperature and pH [4, 23]. Difference spectroscopy has been applied to evaluate the behaviour of tyrosine in such cases [23], as well as during conversion of monomeric insulin to the dimer [5]. But in proteins such as insulin, where Tyr and Phe are found together in the molecule, the zero-order spectrum is not convenient for examining the location of Phe residues because of strong Tyr bands masking weak Phe bands. However, the second-derivative spectrum can be helpful [15, 17]. Thus, if

Table 3
Root-mean squares of differences between *n*th-order derivative spectra for pork and beef insulin in buffered solution, and the corresponding variance ratios relative to system noise

pH	Derivative order	RMS*	*F*-ratio†
7.4	0	0.08502	163909.3
	2	0.02218	11155.4
	4	0.03277	24350.9
	6	0.07173	116671.0
	8	0.18349	763459.9
12.85	0	0.04737	50882.5
	2	0.02135	10336.1
	4	0.0037	310.4
	6	0.03605	29469.4
	8	0.0689	107646.5
	noise	0.00021	

* RMS calculated according to equation (1) (see text).
† *F*-ratio calculated according to equation (2) (see text).

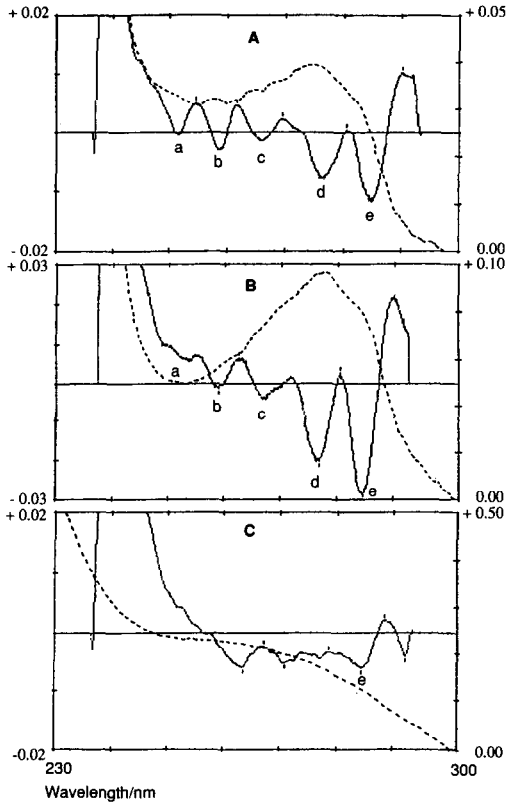

Figure 3
Typical second-derivative and zeroth-order spectra of pork insulin solution (66 μg ml^{-1}, in pH 7.4 phosphate-buffered saline (0.1 M). *a* = 252 nm, *b* = 259 nm, *c* = 267 nm, *d* = 277 nm, *e* = 285 nm. (A) fresh solution; (B) after 48 h and (C) after 144 h oxidative stressing. Instrument: Shimadzu 160A UV–vis spectrometer.

the corresponding second-derivative spectrum of the 48-h air-stressed insulin is examined, it can be seen that, while the derivative absorbance of the minima at 285 and 277 nm have increased, the two minima at 266 and 259 nm have been perturbed and the minimum at 252 nm has completely disappeared. Stability studies on insulin solutions have shown the degradation products to be desamidoinsulins, and high molecular weight polymers which predominate at temperatures above 10°C, being characterized by the same amino acid composition as insulin [9].

In these experiments both insulin solutions formed light-scattering insoluble aggregates, as was evident from the visual opalescence and increased background absorption in the UV spectra. This is indicative of polymerization and the second-derivative spectra show that Tyr and Phe behave in a different manner during this phenomenon. Near-UV CD studies have shown [6–8] that the Zn-containing insulin solutions show a marked increase in CD at 274 nm during hexamerization, which causes restricted rotation of B1 Phe and A14 Tyr, where B16 Phe is completely buried. Although it is not possible to define these observations in such precise terms, these preliminary observations show that derivative spectroscopy may prove to be helpful for studying the behaviour of the aromatic amino acids in the insulin molecule under conditions of oxidative stress, as well as for comparison of insulins from different sources.

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