Effect of Secondary Structures of Protein on Determination of Protein Content by Near Infrared Spectroscopy

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Characteristics of the absorption at 2170 nm, which is due to peptide bonds, were investigated using various proteins. The intensities of the absorption due to peptide bonds were different depending on the kinds of proteins examined. The absorption of bovine serum albumin at 2170 nm decreased by reducing its disulfide (S-S) bonds. The absorption of poly-L-glutamic acid in the α -helix state at 2170 nm was stronger than that in the random coil state. These results suggest that the secondary structures of proteins affect the absorption at 2170 nm. The extent of contribution of the secondary structures to the absorption intensity was statistically predicted using 9 proteins which have different contents in secondary structures such as α -helix, β -sheet and random coil structures. It was found that the relative extent of the α -helix, β -sheet and random coil structures to the absorption at 2170 nm was approximately 2 : 1 : 1.

Introduction

The quantitative determination of various food constituents by near infrared (NIR) spectroscopy has many advantages such as simple operation, rapid measurement and non-destructive analysis, compared with conventional chemical analysis¹⁾. However, NIR spectroscopy has disadvantages from the viewpoint that it is an empirical procedure and needs a number of samples to obtain a stable calibration; that is, a calibration for one item of food cannot be applied to other items. In order to remove such disadvantages, it is necessary to gain fundamental information regarding the NIR absorption. Protein is one of the most popular compositions which can be determined by the NIR spectroscopy. There have been many investigations about the determination of protein content by NIR spectroscopy^{2)~7)}. The most important application is the determination of the protein content of wheat. At present, this method is widely used for routine analysis in many countries⁸⁾⁹⁾. This measurement provides an

accurate determination using a calibration equation developed by multiple regression analysis. However, the wavelengths used for the calibration have not been yet clearly assigned. In our previous report, we investigated the effect of co-existing materials and measuring conditions on the characteristic absorption for determining protein content using bovine serum albumin (BSA) as the model protein, and elucidated that the absorption at 2170 nm due to peptide bonds is the most suitable¹⁰⁾.

In order to apply the absorption at 2170 nm for determining the content of various proteins, it is necessary to investigate how the absorption at 2170 nm is influenced by protein structures. The purpose of this study is to evaluate the absorption intensity at 2170 nm of various proteins and to investigate the effect of the secondary structures of proteins on the absorption intensity around 2170 nm.

Materials and Methods

NIR transmittance measurement

NIR transmittance spectra were recorded

using an NIR Systems (Pacific Scientific) Model 6250 Research Composition Analyzer in wavelength region from 1100 nm to 2500 nm at intervals of 2 nm.

The transmittance measurement was carried out with a quartz glass cell (light path, 1 mm) under 30°C. The spectra were collected as the average value of 50 scans in the wavelength region, and recorded as $\log(1/T)$ which was defined as absorbance (T ; transmittance). A quartz glass cell filled with water was used as the standard reference to calculate $\log(1/T)$.

Materials

Bovine serum albumin (BSA, fraction V), and cytochrome *c* (85–100%) were obtained from Nacalai Tesque (Kyoto, Japan). Ovalbumin (grade V), lysozyme (grade I), myoglobin (95–100%), ribonuclease (Type I-A), chymotrypsin (Type II), subtilisin (Type XXVII), trypsin (Type III) and pepsin (crystallized and lyophilized powder) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Poly-L-glutamic acid sodium salt was purchased from Peptide Institute (Osaka, Japan). All other chemicals were of analytical grade.

Peptide bond content

Protein content was determined by the UV method. The spectrophotometer used was a Hitachi 200–20. The absorption coefficient ($A^{1\%}$ at 280 nm) of each protein was as follows: BSA, 6.6; ovalbumin, 7.35; lysozyme, 26.9; myoglobin, 19; cytochrome *c* 17.1; ribonuclease, 7.1 (at 278 nm); α -chymotrypsin, 20.8; subtilisin, 11.7; pepsin, 13.2; trypsin, 15.4. The peptide bond content (the number of amino acid residues per molar) was calculated on the basis of the primary structure and protein content of each protein.

Reduction of disulfide bond in BSA

The reaction mixture contained 5% BSA and 20 mM dithiothreitol. The reaction was carried out at 30°C in a quartz cell attached to the NIR instrument. The NIR measurement was performed at 10-minute intervals.

Results and Discussion

Relationship between the peptide bond content and the absorption at 2170 nm of proteins

The relationship between the number of amino acid residues (peptide bond molar content) and the value of the second derivative [$d^2\log(1/T)$] around 2170 nm was investigated using BSA, ovalbumin and lysozyme, as shown in Fig. 1. Although high correlations were observed between the peptide bond content and $-d^2\log(1/T)$, the regression coefficient (slope) was different depending on the kind of proteins. In our preliminary experiment, when these proteins were denatured by guanidine hydrochloride (4.2 M), the intensities of the absorptions around 2170 nm due to their peptide bonds were the same (data not shown). However, the absorption intensities around 2170 nm per peptide bond were different among these proteins, suggesting that the absorption around 2170 nm may be affected by the changes in the secondary structures of a protein, not by the changes in their primary structure.

Effect of structure

In order to clarify the reason why the absorption intensity around 2170 nm of each protein was different, we investigated the change of $d^2\log(1/T)$ at 2170 nm caused by the conformational change of protein using BSA as the model protein. The reduction of the disul-

Fig. 1 Relationship between peptide bond content and $-d^2\log(1/T)$ at 2170 nm of proteins

Δ ; ovalbumin, $\square$; lysozyme, $\circ$; BSA

fide bond has been known to cause a change in protein structure¹¹⁾. Accordingly, the disulfide bond of BSA was reduced in the presence of dithiothreitol, and the second derivative spectra of BSA during the reduction were recorded at 10-minute intervals. The spectra are shown in Fig. 2. The absorption at 2170 nm became weaker with the elapse of reaction time. This result suggests that the absorbance at 2170 nm is affected by conformational change in the protein.

Effect of secondary structural change on the absorption at 2170 nm of poly-L-glutamic acid

It has been known that poly-L-glutamic acid has a random coil structure at pH 7.5 and α -helical structure at pH 4.4¹²⁾. In order to investigate how the secondary structural changes affect the absorption at 2170 nm, the second derivative spectra of 1 % poly-L-glutamic acid solutions at pH 7.5 and pH 4.4 were examined (Fig. 3). The absorption intensity at 2170 nm of the polypeptide in α -helical structure (pH 4.4) was much stronger than that in the random coil structure (pH 7.5). This result suggests that the absorption at 2170 nm is significantly affected by the secondary structural change of proteins.

Contribution of secondary structures of proteins to the absorption around 2170 nm

The extent of the contribution of the secondary structure to the absorption around 2170 nm was investigated using nine kinds of proteins such as lysozyme, myoglobin, cytochrome *c*, ribonuclease, chymotrypsin, subtilisin, trypsin, pepsin and ovalbumin. A simple linear regression was run to obtain a correlation between peptide bond content and the $d^2\log(1/T)$ around 2170 nm. The regression coefficients obtained are summarized in Table 1. In order to obtain the extent of contribution of the secondary structures to the $d^2\log(1/T)$ around 2170 nm, the following equation was developed :

$$W_n = a \cdot X_n + b \cdot Y_n + c \cdot Z_n$$

where X_n , Y_n and Z_n are the contents (%) of the α -helix, β -sheet and random coil structures, respectively. W_n is the regression coefficient

Fig. 2 Effect of the reduction of disulfide bond in BSA on the absorption at 2170 nm.

The arrow in the inset shows the elapse of the reducing reaction time (0-60 min).

Fig. 3 Effect of secondary structural change of poly-L-glutamic acid on the absorption at 2170 nm

(a), α -helix (pH 4.4) ; (b), random coil structure (pH 7.5)

Table 1 Relationship between peptide bond content and the $d^2 \log(I/T)$ at 2170 nm

Protein	A ^{a)}	B ^{a)}	R ^{b)}	SE ^{c)}
Lysozyme	3.97	0.0243	-0.992	0.0972
Myoglobin	4.73	-0.00815	-1.000	0.0220
Cytochrome c	3.34	-0.0134	-0.999	0.0262
Ribonuclease	3.09	0.0383	-1.000	0.0161
Chymotrypsin	2.87	0.0109	-1.000	0.0146
Subtilisin	3.53	-0.00564	-1.000	0.0143
Trypsin	2.81	0.0234	-0.999	0.0293
Pepsin	2.49	0.0123	-0.999	0.0324
Ovalbumin	3.23	-0.00662	-0.999	0.0387

a) $-d^2 \log(I/T) = A \cdot (\text{peptide bond content}) + B$

b) Correlation coefficient

c) Standard error

Table 2 Secondary structure content of various proteins

Protein	Secondary structure content (%) [*]		
	α -Helix	β -Sheet	Random coil
Lysozyme	36	41	23
Myoglobin	78	12	10
Cytochrome c	38	17	45
Ribonuclease	24	41	35
Chymotrypsin	10	54	36
Subtilisin	30	30	40
Trypsin	13.45	65.47	21.08
Pepsin	9.8	85.6	4.6
Ovalbumin	30	49	21

^{*} The content was cited from the literatures¹³⁾⁻¹⁶⁾.

(A) in Table 1. The coefficients, a, b and c in the equation are the extent of contribution of the α -helix, β -sheet and random coil structures, respectively. In other words, the relative magnitude of these three coefficients is an index of the extent of contribution of each secondary structure to the absorption at 2170 nm. The nine kinds of proteins of which secondary structures are well known is shown in Table 2 and was used for the calculation. These data were cited from the literatures reported by other researchers¹³⁾⁻¹⁶⁾. The coefficients of a, b and c, estimated by a multiple regression analysis, were 5.42×10^{-2} , 2.34×10^{-2} and 2.64×10^{-2} , respectively. This result

shows that the extent of the contribution of α -helix, β -sheet and random coil structures to the absorption is approximately 2 : 1 : 1, in relative magnitude.

From these results described above, it is concluded that the NIR determination of protein content using the absorption at 2170 nm is affected by the conformational changes in proteins. Therefore, the measurement at 2170 nm could be useful for monitoring the extent of denaturation of proteins in foods during processing and cooking.

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- 近赤外分光法によるタンパク質の定量測定に対する
タンパク質の二次構造の影響
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- 近赤外分光法によるタンパク質含量の測定を様々な種類のタンパク質に適用するために、種々のタンパク質およびポリペプチドを用いて、ペプチド結合に由来する2170 nmの吸収特性について検討した。ペプチド結合数と2170 nmの吸収強度との関係を比較した結果、ペプチド結合当たりの相対吸収強度には、タンパク質の種類により差がみられた。次に、牛血清アルブミンのS-S結合を還元して変性させると、2170 nmの吸収強度が弱くなった。また、pHを変化させることによりポリ-L-グルタミン酸の二次構造を変化させた場合も、2170 nmの吸収強度が変化した。このことから、2170 nmの吸収強度は二次構造により影響を受けると推測された。そこで、二次構造既知のタンパク質を用いて、二次構造含量と2170 nmの吸収強度との関係を統計的に解析した結果、 α -ヘリックス、 β -構造、ランダム構造は、2170 nmの相対吸収強度に対してそれぞれ2:1:1の割合で寄与することが明らかとなった。