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ORIGINAL ARTICLE

A study on the potential interaction between cyclodextrin and lipoxygenase

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Abstract To get a more complete view on the lipoxygenase (LOX) catalysis in presence of cyclodextrin, the investigation into the interaction between cyclodextrins (CDs) and LOX was carried out. Effects of cyclodextrins on the activity and structure of LOX were explored in this work. It is confirmed that inhibition effect induced by complexation of CDs and LOX plays a leading role in inhibition factors of LOX catalysis in presence of CDs. Inhibition of β-cyclodextrin on LOX depended on concentration and tended to be intensified with the increase of β-CD. The enhancement of intrinsic fluorescence of LOX induced by β-CD was detected, which was probably due to the formation of complexes between aromatic amino acid residues of LOX and β-CD. The results of circular dichroism assay indicated that β-CD altered the secondary structure and microenvironment of LOX which was responsible for inhibition of enzyme catalysis.

Keywords Cyclodextrin · Lipoxygenase · Complexation · Microenvironment · Secondary structure

Introduction

Cyclodextrins (CDs) are torus-shaped oligosaccharides composed of glucopyranose units joined by a-(1, 4) bonds. CDs can form inclusion complexes with a great number of organic or inorganic compounds due to their hydrophobic internal cavities and hydrophilic external rims [1, 2]. Their special spatial conformation contributes to various interesting characteristics and applications. Owing to the peculiar spatial arrangement, CDs possess the potential to form supermolecular cyclodextrin-protein complexes, of which is been used for stabilization of proteins against aggregation and to prevent off undesired flavors induced by lipoxygenase (LOX) during the preservation of foods. Giani [3] reported that β-CD complexation is effective for modifying single amino acids taste perception and debittering Soy protein hydrolysate. However, there are no systematic reports to clarify the fundamentals and the role of CDs in LOX catalysis. Moreover, studies mainly focus on attributing the inhibition to the combination of substrate and CDs [4]. The characteristics of the interaction between CDs and LOX remain to be clearly elucidated.

Owing to the peculiar spatial arrangement, CDs possess the potential to form supermolecular cyclodextrin-protein complexes. The influence of CD on enzymes was studied by several researchers [5–7]. Moreover, the interactions of CD and proteins [5, 8–10] were investigated and ideal results have been obtained. Aachmann et al. [11] reported that the interaction of β-CD and their model proteins takes place at specific sites on the protein surface. Furthermore, numerous studies have demonstrated the interactions between CD and peptides [5, 12–14], and isolated amino acids [15–19]. The focus of the present investigation is to study the interaction of CDs with LOX and illustrate the mechanism explicitly. Soy LOX-1 was chosen as the model in our experiments owing to its known crystal structure.

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Materials and methods

Material

β-CD, linoleic acid (LA, 99 % purity) and soybean LOX type-1 (100,000 U/mg) in freeze-dried powder, were purchased from Sigma-Aldrich Trading Co., Ltd (Shanghai, China). α-CD, γ-CD were purchased from TCI, Japan; H_3BO_3, $Na_2B_4O_7{\cdot}10H_2O$,HCl, NaOH, and EtOH were of analytical grade. Tween 20 and all other chemicals and reagents were of reagent grade. HPLC grade water was used in all experiments.

Preparation of linoleic acid solutions

Tween 20 (1.3 ml) were dispersed in 20 ml of borate buffer solution (pH 9.0) and then linoleic acid (1.5 ml) was added by dropwise with shaking. Then 11 ml of 0.5 N NaOH was added to increase the optical clarity. And then the mixture was adjusted to pH 9.0 with 0.1 M HCl and the final concentration of linoleic acid stock solution was set to 10 mM with sodium borate buffer. The substrate solution was prepared and kept at 4 °C prior to use.

Lipoxygenase assay

LOX-1 activity was determined by following the increase in absorbance at 234 nm due to the formation of hydroperoxide. The reaction mixture contained 60 μl of linoleic acid (10 mM), 0.5 ml of LOX-1 (0.238 mg/ml) and 2.44 ml of sodium borate buffer (0.2 M, pH 9.0), to make a total volume of 3 ml. The reaction was stopped by the addition of 5 ml EtOH. One unit activity is defined as an increase in absorbance at 234 nm of 0.001/min at 25 °C and pH 9.0. A TU-1900 double beam UV–Vis spectrophotometer (Pgeneral, Beijing, China) was used for all assays.

Inhibition of lipoxygenase activity

The experiment was carried out with β-CD, the most commonly used CD, at 25 °C in sodium borate buffer (0.2 M, pH 9.0). Each assay had a total volume of 3 ml. β-CD was dissolved in sodium borate buffer. Sodium borate buffer alone was added in uninhibited controls.

Percent enzyme inhibition was expressed as $100 \times (A_1 - A_2)/A_1$ where A_1 and A_2 are the values for the rate of absorbance increase in blanks (with sodium borate buffer alone added) or for test samples (with β-CD added), respectively.

The following experimental groups were set through changing the add order of β-CD (10 mM), LOX-1 (0.238 mg/ml) and linoleic acid (10 mM).

(a) Substrate was added after the mixture of β-CD and LOX-1 was incubated for 1 h.
(b) Substrate, β-CD and LOX-1 were added simulta neously.
(c) LOX-1 was added after the mixture of β-CD and substrate was incubated for 1 h.

For the experiments, the LOX-1 was kept on ice and the controls were determined at intervals to make sure the constancy of enzyme activity. The reactions above were all stopped by the addition of EtOH. All the experiments were average of at least six determinations.

Factors that affect the CD–LOX assay

Type of CD

All the above experiments were performed with β-CD. In further experiments α-CD, γ-CD were tested at the same reaction condition as β-CD in the inhibition assay above.

CD concentration

β-CD was dissolved in sodium borate buffer and assayed at different concentrations (1.5, 3.0, 4.5, 6.0, 7.0, 8.0, 9.0, 10.0, 12 mM); LOX-1 concentration was 0.238 mg/ml. Sodium borate buffer alone was added in uninhibited control experiments. In these experiments, the mixture of β-CD and LOX-1 was incubated for 1 h and then linoleic acid was added. At least six parallels were run at each concentration of test substance.

Fluorescence measurements

Fluorescence spectra were obtained with an F-7000 fluorescence spectrometer (Hitachi, Japan), using $\lambda_{ex} = 280$ nm, $\lambda_{em} = 290$–450 nm, 5.0 nm excitation and 5.0 nm emission slit widths, and 12,000 nm/min of scanning speed. Appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence.

Circular dichroism measurements

Circular dichroism spectra were recorded on MOS-450 Chiral Detector (Biologic Science Instruments, Grenoble, French) using a quartz cuvette with a 0.1 cm optical pathlength in the far-UV range (190–250 nm) at 25 °C. Instrument baselines were measured using sodium borate buffer (0.2 M, pH 9.0). The circular dichroism data was obtained through four parallels. Scans were reported as mean molar ellipticities ([θ], degree cm^2/dmol). The content of secondary structure was calculated with the method of K_2D.

Results and discussion

Inhibition of lipoxygenase activity by β-CD

In order to demonstrate the interaction of CD and LOX, three test groups (a, b, c) were set through by altering the adding sequences of β-CD. Figure 1 shows the percentage inhibition varies with adding order for the three groups (a, b, c). Comparison between different test groups indicates group of adding substrate after the incubation of β-CD and LOX-1 for 1 h causes the maximal inhibition. The results illustrate the enzyme apparent inhibition by CD is due to complexation of β-CD and LOX-1 except substrate-CD combination. Inhibition effect induced by complexation of CDs and LOX is more significant than inhibition effect resulted from inclusion of CDs and linoleic acid. Inhibition caused by complexation of CDs and LOX plays a leading role in inhibition effect of LOX catalysis in presence of CDs. The assay brings out the conclusive evidence on the interaction of β-CD and LOX-1.

Factors that affect the CD–LOX assay

Type of CD

In order to study the effect of cavity size of CD on inhibition, the inhibition behavior of α-CD and γ-CD was observed. Different size of CD exhibit varying influence on enzyme activity as Fig. 2 shown. With α-CD, β-CD and γ-CD adding into the model inhibition reaction system (LOX+CD→LA), percent enzyme inhibitions were 70.78 %, 86.26 %, 66.22 %, respectively. The results indicate the hydrophobic cavity of β-CD may match with LOX-1 best, followed by α-CD, then γ-CD. That is, β-CD possesses the optimal spatial adaptation to the structure of LOX-1.

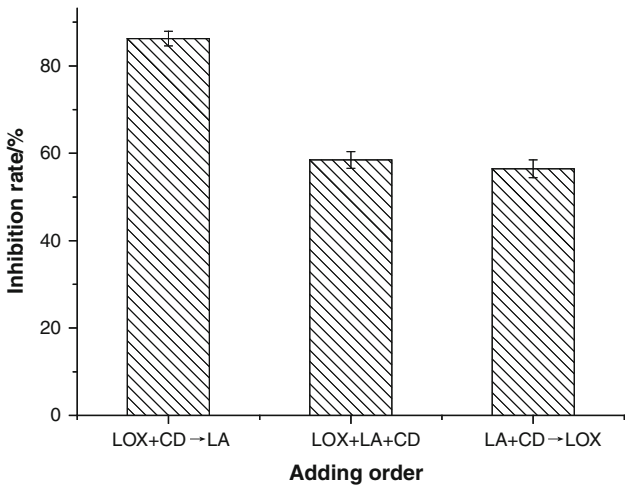

Fig. 1 Inhibition assay through changing the add order of β-CD, LOX-1and LA. LOX + CD → LA represent the test group that substrate was added after the mixture of β-CD and LOX-1 were incubated for 1 h; LOX + LA + β-CD represent the test group that substrate, β-CD and LOX-1 were added simultaneously; LA + β-CD → LOX represent the test group that LOX-1 was added after the mixture of β-CD and substrate were incubated for 1 h

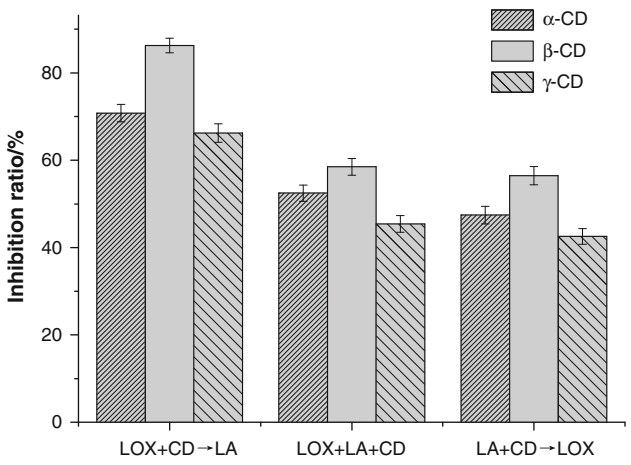

Fig. 2 Effects of type of CD on LOX activity under the three adding order as Fig. 1. The concentrations of α-CD, β-CD and γ-CD are all 10 mM

CD concentration

In the interest of reveal the effect of β-CD on LOX-1, the relationship between LOX-1 activity and the concentration of β-CD has been determined. Increasing concentrations of β-CD (1.5, 3.0, 4.5, 6.0, 7.0, 8.0, 9.0, 10.0, 12 mM) have been added into the model reaction system. As shown in Fig. 3, percent enzyme inhibition presented the gradually increasing tendency with increasing amounts of β-CD and then tended to be steady. Thus an inhibitory effect concentration dependent is detected, with 10 mM β-CD causing approximately 86 % inhibition (Fig. 3). As is well known, the decrease in enzyme activity arises from conformational modification of enzyme. Accordingly, so as to probe into inhibition mechanism of β-CD on LOX-1 catalysis, alterations of LOX-1 microenvironment and secondary structure were detected by fluorescence spectra and circular dichroism spectra.

Fluorescence measurements

Fluorescence spectroscopy has been widely used as a powerful tool to evaluate changes in the secondary structure of enzyme. It is an effective technique to provide valuable information about the accessibility of ligands to proteins (fluorophores) [20]. It has been reported that the intrinsic fluorescence of LOX is due to the aromatic aminoacids, mainly tryptophan, phenylalanine and tyrosine [21]. With the aim to obtain a more complete view about the interaction of CD with LOX-1, fluorescence

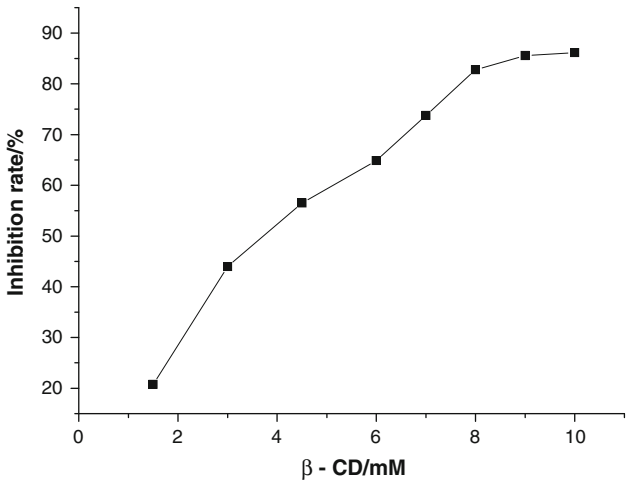

Fig. 3 Effects of the concentration of β-CD on LOX activity

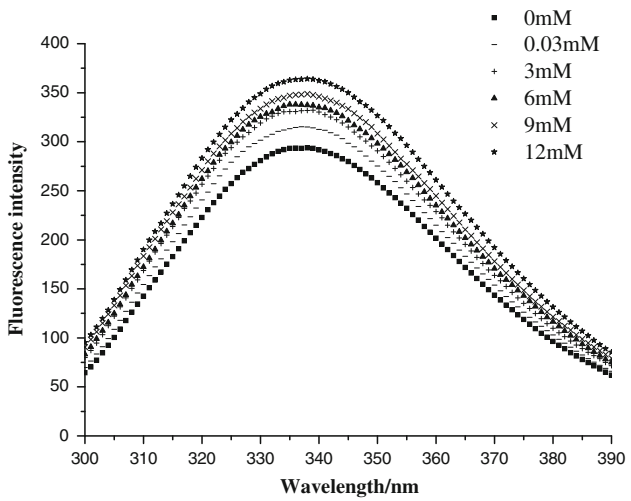

Fig. 4 Effects of β-CD on intrinsic fluorescence of lipoxygenase in the absence of β-CD (■) and in the presence of β-CD: 0.03 mM (−), 3 mM (+), 6 mM (▲) , 9 mM (×) and 12 mM (★). Each data point was the mean of three replicates

measurements were carried out. Figure 4 shows the fluorescence emission spectra of LOX-1 in the presence of β-CD concentrations varying from 0.0 to 12.0 mM. When LOX is excited at 280 nm, a broad emission band centered at 330–340 nm induced by the emission of Tyr and Trp residues is obtained. As shown in Fig. 4 that increasing concentrations of β-CD lead to a gradual increase of fluorescence intensity with modification of the emission maximum. The florescence intensity was 293.8 units for LOX in absence of β-CD, increasing to 364 units (a 24 %) in the presence of β-CD (12.0 mM).

CDs coupling with analyte molecules (chromophore) can result in significant enhancement of the fluorescence. Primary causations consist in that the structural conformation of the CD can protect the fluorescing singlet state and afford an apolar surrounding to enhance quantum yield [22].

There is a high probability that β-CD complexes with the amino acids constitute the catalytic center and the access channel for the substrate to the catalytic site [23]. This region, primarily comprised of hydrophobic amino-acids (Trp340, Phe346, Gly353, Ala542, Leu546, Glu697, Leu754) [23] is highly conserved for LOX from soybean and contains a Trp residue [21] which is the main fluorophore residue responsible for the intensifying fluorescence.

From the results above, it can be concluded that β-CD forms a complex with LOX-1 at some specific sites of the enzyme and during the process hydrophobic interactions are involved. The interaction may result in slight alteration of enzyme conformation and the microenvironment of the binding sites, consistent with with our previous studies.

Circular dichroism measurements

Circular dichroism is the most commonly used approach to analyze secondary structure of proteins. The contents of α-Helix, β-Sheet and random coil can be analyzed and calculated from the circular dichroism spectra in far-UV region by means of K_2D method [24–26]. The CD assay was performed with increasing concentration of β-CD. As can be seen in Fig. 5, as the concentration of β-CD increases from 0 to 10 mM, the percentage of α-helix gradually increased from 36.07 to 41.23 %. On the other hand, β-Sheet content decreased from 17.85 to 13.76 %. Meanwhile, β-turn content slightly decreased from 18.74 to 14.84 % and random coil from 27.34 to 30.17 %, respectively. This finding reveals that β-CD induces structural changes of LOX-1. It is more likely that β-CD entrapped hydrophobic amino acid residues of LOX-1 into its cavity and consequently modify the amino acid side chains and

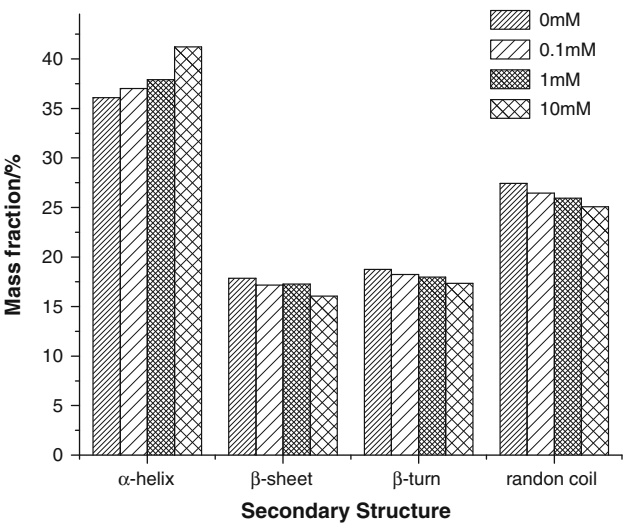

Fig. 5 Effects of β-CD on secondary structure of LOX in the absence of β-CD and in the presence of β-CD: 0.1, 1, and 10 mM. Each data point was the mean of three replicates

secondary structure content. This finding holds with the fluorescence assay involved in conformational variation.

Conclusions

In the present study, the inhibition mechanism of β-CD on LOX was investigated by exploring structure and microenvironment changes. We have presented detailed data to confirm that β-CD caused the loss of enzymatic activity significantly and the effect was concentration-dependant to some extent. Moreover, the inhibition of β-CD on LOX-1 catalysis is attributed to the formation of the potential β-CD–LOX-1 complex rather than to the β-CD–LA inclusion. The fluorescence spectroscopy showed inclusion formed between β-CD and aromatic aminoacids, which provided the evidence that the peculiar CDs hydrophobic cavum of β-CD caused a conformational change of LOX molecule. The circular dichroism spectroscopy demonstrated that the secondary structure of LOX was induced by β-CD in a concentration depended manner as well. The ratio of α-helix, β-sheet and random coil changed in the presence of β-CD, thus resulting in the loss of enzymatic activity. Therefore, it is tempting to speculate that the inhibition of LOX induced by β-CD was related to the modification of structure and microenvironment. This knowledge might be used to supplement inhibition mechanism of β-CD on LOX.

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References

1. Martin Del Valle, E.M.M.: Cyclodextrins and their uses: a review. Process. Biochem. **39**, 1033–1046 (2004)
2. Szejtli, J.: Introduction and general overview of cyclodextrin chemistry. Chem. Rev. **98**, 1743–1752 (1998)
3. Giani A. L.: The use of 2D NMR to study β-cyclodextrin complexation and debittering of amino acids and peptides. Food. Res. Int. **43**(1), 187–192 (2010)
4. J. M. López-Nicolás, Bru, R., García-Carmona, F.: Kinetic characteristics of the enzymatic conversion in presence of cyclodextrins: study of the oxidation of polyunsaturated fatty acids by lipoxygenase. Biochim. Biophys. Acta. **1347**, 140–150 (1997)
5. K. Guzow, W. Wiczk.: Interactions of cyclodextrins with amino acids, peptides and proteins. In: Fitzpatrick, D.W., Ulrich, H.J. (eds.) Macrocyclic Chemistry: New Research Developments, pp. 233–262. Nova Science Publishers, Inc., New York (2010)
6. Sharma, L., Sharma, A.: Influence of cyclodextrin ring substituents on folding-related aggregation of bovine carbonic anhydrase. Eur. J. Biochem. **268**(8), 2456–2463 (2001)
7. Yu, B., Tian, Y., Yang, N., Xu, X., Jin, Z.: A study on the inhibition mechanism of β-cyclodextrin on pullulanase. J. Incl. Phenom. Macrocycl. Chem. **70**, 161–165 (2011)
8. Yu, B., Wang, J., Zhang, H., Jin, Z.: Investigation of matching effect between hydrophobic cavities of cyclodextrins and pullulanase. Molecules **16**(4), 3010–3017 (2011)
9. Samra, H.S., He, F., Bhambhani, A., Pipkin, J.D., Zimmerer, R., Joshi, S.B., Middaugh, C.R.: The effects of substituted cyclodextrins on the colloidal and conformational stability of selected proteins. J. Pharm. Sci. **99**(6), 2800–2818 (2010)
10. Serno, T., Geidobler, R., Winter, G.: Protein stabilization by cyclodextrins in the liquid and dried state. Adv. Drug. Deliv. Rev. **63**(13), 1086–1106 (2011)
11. Aachmann, F.L., Otzen, D.E., Larsen, K.L., Wimmer, R.: Structural background of cyclodextrin-protein interactions. Protein. Eng. **16**(12), 905–912 (2003)
12. Lula, I., de Sousa, F.B., Denadai, A.M.L., Ianzer, D., de Camargo, A.C.M., Santos, R.A.S., Sinisterra, R.D.: Study of the BPP7a peptide and its β-cyclodextrin complex: physicochemical characterization and complete sequence specific NMR assignments. J. Braz. Chem. Soc. **22**(9), 1765–1773 (2011)
13. Kanegae, H., Morii, H., Harata, K.: Design and synthesis of cyclodextrin-peptide conjugates and their characterization. Pept. Chem. **34**, 477–480 (1996)
14. Gere-Paszti, E., Cserhati, T., Forgacs, E., Deyl, Z., Miksik, I., Eckhardt, A., Illes, Z.: Interaction of hydroxypropyl-β-cyclodextrin with peptides, studied by reversed-phase thin-layer chromatography. J. Liq. Chromatogr. Relat. Technol. **28**(16), 2619–2632 (2005)
15. Ignash, M., Ong, L., Freel, K., Chai, M.: (2005) NMR investigation on the interaction of cyclodextrin and amino acids. In: Abstracts of Papers, 229th ACS National Meeting, San Diego, CA, March 13–17
16. Ciesielski, W., Kapusniak, J.: Interactions of amino acids with β-cyclodextrin and with potato starch studied by thermogravimetric measurements. J. Incl. Phenom. Macrocycl. Chem. **64**(1–2), 109–114 (2009)
17. Shanmugam, M., Ramesh, D., Nagalakshmi, V., Kavitha, R., Rajamohan, R., Stalin, T.: Host–guest interaction of L-tyrosine with β-cyclodextrin. Mol. Biomol. Spectrosc. **71A**(1), 125–132 (2008)
18. Bikadi, Z., Ivanyi, R., Szente, L., Ilisz, I., Hazai, E.: Cyclodextrin complexes: chiral recognition and complexation behavior. Curr. Drug. Discov. Technol. **4**(4), 282–294 (2007)
19. Hashidzume, A., Harada, A.: Macromolecular recognition by cyclodextrins. Interaction of cyclodextrins with polymethacrylamides bearing hydrophobic amino acid residues. Polymer **47**(10), 3448–3454 (2006)
20. Lakowicz, J.R.: Principles of Fluorescence Spectroscopy, 3rd edn. Kluwer/Publishers, New York (2008)
21. Burstein, E.A., Vedenkina, N.S., Ivkova, M.N.: Fluorescence and the location of tryptophan residues in protein molecules. Photochem. Photobiol. **18**, 263–279 (1973)
22. Li, S.: Cyclodextrins and their applications in analytical chemistry. Chem. Rev. **92**, 1457–1470 (1992)
23. Ivanov, I., Heydeck, D., Hofheinz, K., Roffeis, J.: Molecular enzymology of lipoxygenases. Arch. Biochem. Biophys. **503**, 161–174 (2010)
24. Spaapen, L.J.M., Veldink, G.A., Liefkens, T.J., Vliegenthart, J.F.G., Kay, C.M.: Circular dichroism of lipoxygenase-1 from soybeans. Biochim. Biophys. Acta **574**, 301–311 (1979)
25. Ludwig, P., Tordi, M.G., Colosimo, A.: Circular dichroism observations on the lipoxygenase from reticulocytes. Biochem. Biophys. Acta **830**, 136–139 (1985)
26. Provencher, S.W., Glockner, J.: Estimation of globular protein secondary structure from circular dichroism. Biochemistry **20**, 33–37 (1981)