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Circular Dichroism as a Process Analytical tool to monitor the Quality of Serratiopeptidase

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ABSTRACT

Circular Dichroism is a sensitive yet simple analytical tool for analysis of protein conformation and structural changes which can occur due to untoward process changes and handling. Serratiopeptidase, produced by fermentation is a proteolytic enzyme which is used as an anti-inflammatory drug. The isolation of pure serratiopeptidase from fermentation broth involves a number of unit operations during its manufacturing. Taking into account a variety of processing and storage during its manufacture; the objective of this study was to determine whether these variations lead to changes in the secondary structure of serratiopeptidase and hence loss in its biological activity. The impact of manufacturing changes on structure and activity of serratiopeptidase during the development was assessed by carrying out three comparative studies. Structural variability was observed when there was drastic variation in the conditions of manufacturing. Thus the data presented here illustrates the use of circular dichroism as quick process analytical tool which can be used to monitor the manufacturing process qualitatively and assure the quality of the product. The comparative quantitative analysis of serratiopeptidase as against circular dichroism was carried out by well established techniques such as HPLC and caseinolytic assay.

KEYWORDS**: **Serratiopeptidase, circular dichroism, HPLC, caseinolytic assay, process analytical tool

INTRODUCTION

Serratiopeptidase is an endopeptidase with a molecular weight of about 52 kDa and has EC number 3.4.24.40. It is widely used as an anti-inflammatory drug to combat inflammation and inflammatory disorders [1]. Being a proteolytic enzyme, it digests dead tissues, cyst, and build-up mucus and thus helps in many ailments. Serratiopeptidase is prescribed in surgery, dentistry, orthopaedics for its anti-inflammatory, analgesic and anti-endemic effects [2]. Serratiopeptidase has proved to be a superior alternative to traditional NSAIDS having pronounced side effects, which are prescribed to treat osteoarthritis, rheumatoid arthritis and other related disorders. Serratiopeptidase also referred as serrapeptase has been prescribed for treating chronic sinusitis, carpel tunnel syndrome, torn ligaments and post operative inflammation [3-5]. Due to its caseinolytic and fibrinolytic properties, it plays an important role in controlling atherosclerosis [6].

Serratiopeptidase is produced by enterobacterium *Serratia marcescens* which is isolated and purified from its fermentation broth. The isolation of serratiopeptidase is carried out by either ‘solvent precipitation using acetone’ or ‘spray drying’. During manufacturing the enzyme is exposed to rigorous and varied processing,

handling and storage. Various analytical methods have been reported in the literature like UV spectroscopy; ELISA, HPLC, LCMS, derivative absorption spectroscopy, x-ray diffraction and caseinolytic assay but none of the publications have utilized circular dichroism technique as an analytical tool for end product or in-process analysis of serratiopeptidase [7].

Characterisation of proteins is useful not only in proteomics but also in pharma manufacturing of large molecules. CD spectrum is a valuable tool in studying protein structure as conformational motifs such as α-helix, β-sheets and turns have characteristic CD spectra [8-9]. The spectrum of the protein is the sum of these characteristic motifs and hence can be used to determine the secondary structure of the protein. Thus CD can be used to monitor the conformational changes which can occur when protein is exposed to factors such as heat, pH, denaturant and shear force [10-12]. The variability in any parameter during manufacturing can hence be quickly ascertained qualitatively by comparing the CD spectra of in-process batch against a control batch. CD as a process analytical tool has not been utilized to assure the quality of serratiopeptidase to date. Here we determined some of the factors which were relevant in manufacturing process of serratiopeptidase that could affect protein structure by CD. This was supported by performing the existing methodology used in the process viz., HPLC and caseinolytic activity determination.

MATERIALS AND METHODS

Samples

All samples of serratiopeptidase were obtained from Ipca Laboratories Limited. The samples were prepared in demineralised water for HPLC, while in buffer for enzymatic assay and CD studies. The buffer pH was adjusted accordingly from pH 1 to pH 11 in the pH study of serratiopeptidase. The concentration used for HPLC and CD studies was 5mg/mL, while for enzymatic hydrolysis was 5µg/mL. No precipitation was observed and samples dissolved completely in the respective diluents.

HPLC

Analytical grade sodium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium hydroxide and HPLC grade acetonitrile were procured from Merck Life Science India Limited. Syringe filters from Millipore of 0.45µ were used to filter the samples. Analysis was performed using Waters Alliance HPLC with UV detector hooked to Empower version 3.0. Separation was carried out on Biosep s2000 column having 300 mm length with 7.8 mm ID. The mobile phase consisted of mixture of phosphate buffer pH 6.5 and acetonitrile in 95:5 proportions respectively. The flow rate was 0.5 ml /min and column was thermo-stated at 25^{o}C. The injection volume was 50 µL and the output was monitored at 220 nm wavelength.

Enzymatic hydrolysis

The enzymatic hydrolysis was performed exactly as described in ‘Serratiopeptidase’ monograph of Indian Pharmacopeia [13].

CD

Serratiopeptidase far-UV CD spectra were collected on a Jasco J-1500 circular dichroism spectrometer. Far-uv spectra were obtained at 250-190 nm with 1 nm bandwidth and temperature controlled at 25 $\pm$ 1^{o} C. The Far-UV CD samples were tested at 0.5 mg of serratiopeptidase /mL in quartz cells with a path length of 0.1 cm.

RESULTS AND DISCUSSION

The function of proteins is intrinsically related to their structure and structural analysis of proteins is important in determining its biological activity. The abundance ratio of secondary structure motifs such as α-helix, β-sheet, turn and random coil can be estimated by CD spectroscopy. The JWSSE-513 program of protein secondary structure analysis as per Jasco includes the reference spectra of Yang [14]. Yang’s reference spectra are extracted from CD spectra of proteins and are best suited to protein secondary structure analysis.

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To quantify the variance between the serratiopeptidase samples, samples obtained by regular manufacturing process (solvent precipitation to isolate serratiopeptidase) and spray drying were used as source material for the study. Serratiopeptidase samples were diluted to 0.5mg/mL in water and were analysed using CD spectroscopy for different batches, while dissolved in respective buffers for pH study. We observed no variation in the CD spectra of the samples isolated from solvent precipitation and spray drying of serratiopeptidase. Similarly other regular batches with different activities were analysed by CD spectroscopy to monitor the manufacturing process but no variations were observed in CD spectra suggesting proper control of manufacturing and uniformity in the process. There was change in the absorbance which was related to the change in the biological activity of serratiopeptidase, either more or less than the reference spectra of standard sample. The difference in the spectra was observed when there was a change in the pH of the concentrated solution of serratiopeptidase which was used for solvent precipitation. The decrease in the pH to pH 3.0 or less led to complete destruction of secondary structure of serratiopeptidase viz.; α-helix and β-sheet motif to turn and random coil. The changes in the secondary structure of serratiopeptidase led to its disordered state and hence loss of its biological activity. This proved that the conformation of serratiopeptidase was linked with its α-helix and β-sheet motifs. Refer figure 1 and table 1 for the pH study of serratiopeptidase. The temperature variations during drying after solvent precipitation which led to decrease in the activity of serratiopeptidase were reflected in the CD spectra. This was supported by comparative studies for determination of enzyme activity by HPLC and enzymatic hydrolysis.

Figure 1: CD spectra of serratiopeptidase at different pH

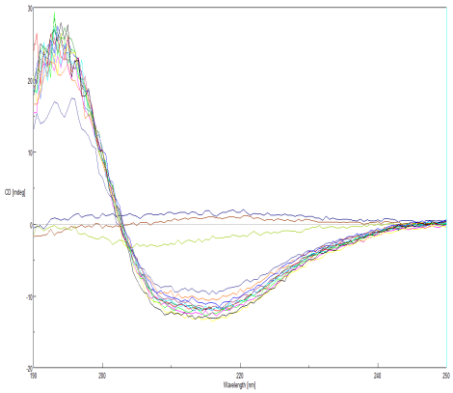

Table 1: CD data at different pH of serratiopeptidase

pH	Colour trace	α-Helix	β-Sheet	turn	Random coil
As such*	Dark green	45.6	46.9	0	7.5
1.0	Navy blue	0	0	100	0
2.0	Brown	0	0	83.5	16.5
3.0	Pale green	4.7	55.8	6.1	33.4
4.0	Blue	32.8	51.4	2.4	6.4
5.0	Orange	42.5	49.9	0	7.6
5.5	Cyan	40.4	51.1	0	8.5
6.0	Purple	45.0	47.4	0	7.6
6.5	Parrot green	44.0	45.8	0	10.2
7.0	Pink	37.0	49.3	0	13.7
7.5	Light blue	49.5	47	0	3.5
8.0	Yellow	33.7	53.8	0	12.5
9.0	Red	42.9	47.8	0	9.5
10.0	Neon blue	39.3	50.9	0	9.7
11.0	Black	380	49.6	0	12.4

*pH 6.8

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The comparison of the results obtained from HPLC activity analysis and biological activity analysis against the secondary structure analysis by CD was done in order to prove the concurrence between the results observed. The compilation of results of HPLC, biological activity and percentage of secondary structure is presented in the table 2. The corresponding CD spectra of these samples are shown in figure 2. The colour traces of samples which are above the control sample trace are having less activity than control sample trace while the traces which appear below the 'control sample trace' are having more activity. This shows that CD analysis is a useful qualitative tool to monitor manufacturing process of serratiopeptidase, which is very simple, straightforward technique. The analysis is quick to perform and requires very small amount of sample.

Figure 2: Overlay CD spectra of different manufacturing batches

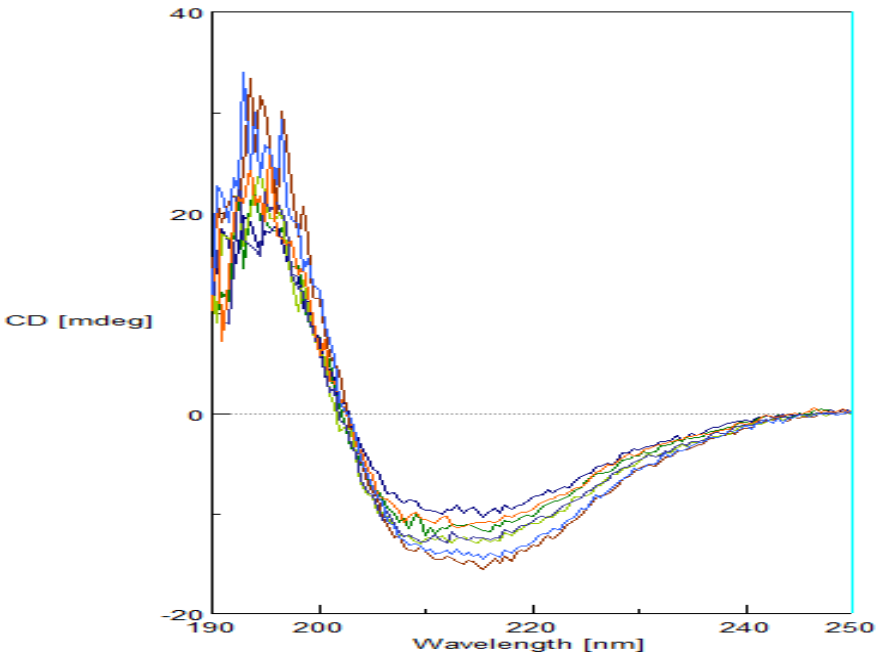

Table 2: The results obtained for different batches of Serratiopeptidase by HPLC, biological assay and CD spectra

B. no	Colour trace	Caseinolytic Activity in units/mg	HPLC activity in units/mg	% of Secondary structure motifs of protein by Yang's spectra [14]			
				α-Helix	β-Sheet	Turn	Random
01	Navy blue	2462	2407	32.7	56.8	0	10.5
02*	Orange	2766	2675	27.6	56.1	3.5	12.7
03	Dark green	2867	2818	25.5	60.8	0	13.8
04	Light blue	2923	2850	21.7	58.3	3.6	16.4
05	Pale green	2921	2886	23.9	58.8	0	17.3
06#	Cyan	2967	2875	35.7	48.5	4.5	11.3
07#	Brown	3572	3400	30.0	52.6	5.1	12.3

*Control batch, # Spray dried at 50 ^{o}C batches

Table 3 presents the data on the effect of temperature on serratiopeptidase. On heating at 40^{o} C there is a very slight loss in the activity of serratiopeptidase. The sample (# Working Standard) was kept for 2 days at 40^{o} C to authenticate the drying temperature used in the manufacturing. Serratiopeptidase is dried at temperature 35 $\pm$ 3^{o} C. The comparative CD spectra are presented in figure 3.

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Figure 3: CD spectra of serratiopeptidase exposed to 40° C

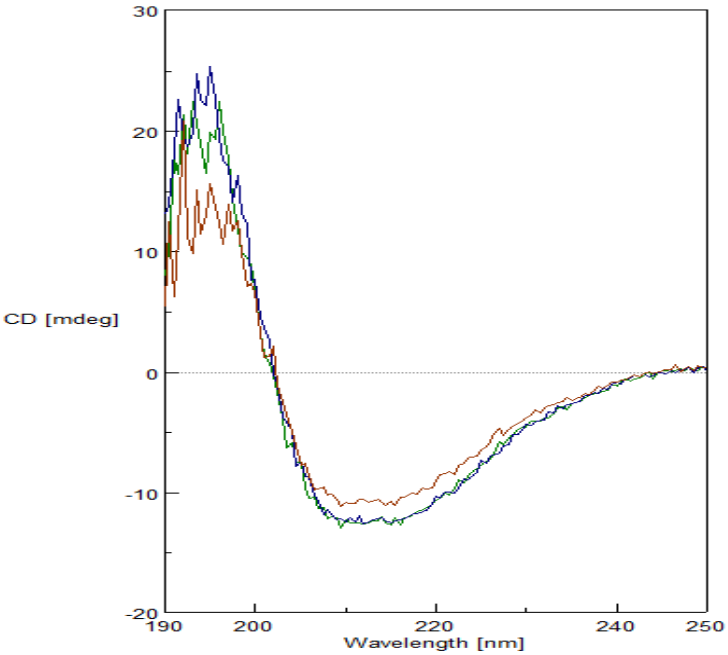

Table 3: Comparison of results after heating serratiopeptidase at 40°C and spray drying

B. no	Colour trace	Caseinolytic Activity in units/mg	HPLC activity in units/mg	% of Secondary structure motifs of protein by Yang's spectra			
				α-Helix	β-Sheet	Turn	Random
Working standard	Green	2700	2765	24.2	60.1	0	15.7
Working standard at **40°C**	Blue	2650	2700	27.4	59.1	0	13.5
08$	Brown	2600	2685	21.5	55.8	5.3	17.4

$: normal batch

CONCLUSION

On the basis of the studies conducted by CD on serratiopeptidase and its comparison to established manufacturing release analysis, we can justify the use of CD as a quick process analytical tool. This can be used like Raman spectroscopy or NIR spectroscopic analytical process technologies which are in use by pharmaceutical industry as qualitative identification tools. Similarly CD spectroscopy can be utilized for the conformational analysis during manufacturing of other enzymes as well.

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