International Journal of Pharma and Bio Sciences V1(1)2010

EFFECT OF ENZYMES ON EXTRACTION OF ANDROGRAPHOLIDE FROM *ANDROGRAPHIS PANICULATA* NEES.

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ABSTRACT

Andrographis paniculata (Family: Acanthaceae) is used- for its bitter tonic, stomachic, antipyretic and laxative properties in ayurveda. The present study consists of utilizing various enzymes and their combination to facilitate the release of andrographolide from polymeric cell matrix, which in turn will facilitate extraction of andrographolide from *Andrographis paniculata.* .Cellulase and Amylase enabled extraction of 0.37% and 0.34% of andrographolide. Cellulase was found to be most effective in releasing andrographolide as compared to other enzymes and enzyme blend.

KEYWORDS

Enzymes, Extraction, Andrographolide, *Andrographis paniculata Nees.*

INTRODUCTION

Andrographis paniculata commonly known as "king of bitters" is used for its bitter tonic, stomachic, antipyretic and laxative properties in ayurveda. It is said to increase appetite, strengthen digestion and diminish flatulence and hyperacidity. The herb is utilized for the treatment of many conditions including diabetes, debility and hepatitis. In fact it is widely used as a hepatoprotective drug [1]. The roots and leaves have the reputation for being anthelmentic [2]. Andrographolide is used as an immunomodulator [3] antibacterial [4,5,6], anti-inflammatory [7].

Major active constituent of *Andrographis paniculata* is andrographolide, a diterpene lactone. It is present in 0.5-0.9 % yield. Due to its high potential use as a hepatoprotective drug there is a need to extract and isolate andrographolide. Conventional modes of extraction (e.g. digestion, soxhlet extraction, etc.) use heat treatment, which is less capable of breaking the cell matrix; while in chemical treatment polysaccharide matrix can be broken by acid hyrdrolysis, but there are chances of degradation of active constituents and energy consumption. However the enzymatic cell disruption can be a best method for extraction. With the use of enzymes and their combination will facilitate the release of

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andrographolide from polymeric matrix, which in turn will facilitate the release and extraction of andrographolide from *Andrographis paniculata Nees.*

MATERIAL AND METHODS

2.1. Standardization and dose optimization of enzymes:

2.1.1. Amylase:

The amount of amylase required to act on standard substrate was determined by the following method, which depends on the ability of the enzyme to hydrolyze the starch. 100mg of amylase was accurately weighed and dissolved in sufficient acetate buffer (pH=5.0) and the volume was made to 100 ml. Cornstarch (200 mg) used as standard substrate was accurately weighed and added into sufficient distilled water (warm).It was then further warmed gently with stirring and volume was made to 100 ml. The final concentration obtained was 2 mg/ml.

2.1.1.1. Method:

5 ml of standard solution of starch (10 mg) was added into each of 15 test tubes, which were initially numbered serially. The tubes were placed in water bath, which was maintained initially at 40°C. When temperature of the solution in the test tubes was reached 40°C; blank, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 ml amylase solution was added into the test tubes from test tube number 1 to 15 respectively. The content in the tubes were mixed thoroughly and placed in water bath (40°C) to maintain the temperature. After 60 minutes, the tubes were removed and in each test tube 0.05 ml of 0.02 M iodine solution was added. The content of the tubes were mixed thoroughly. The tubes were observed for the presence of blue colour. Minimum 4 mg of given enzyme was required for the complete digestion of 10 mg substrate. Thus the quantity of enzyme required was 40% of the substrate quantity.

2.1.2. Cellulase:

The amount of cellulase required was based on the principal that cellulase hydrolyses carboxy methylcellulose and produce carboxy methyl oligosaccharides; which on reacting with 3,5, dinitrosalysilic acid (3,5-DNS) produced red color which is measured at 546 nm. 500 mg of sodium salt of carboxymethyl cellulose was dissolved in 100 ml acetate buffer (pH=5). The concentration of the substrate was 5 mg/ml. 200 mg of cellulase was weighed and added into the acetate buffer. Final volume was made to 100 ml with acetate buffer.

2.1.2.1. Method:

4 ml Na-CMC solution was added into each 15 test tubes, which were initially numbered. Precaution was taken not to touch the solution on the sidewalls of the test tubes. In these test tubes 0, 0.2,0.4,0.6,0.8,1.0,2,3,4,5,6,7,8,9 and 10 ml enzyme solution was added from test tubes number 1-15 respectively. Tubes were put on a rotary shaker for 24 hour. After 24 hour test tubes were removed and the volume was made to 15 ml in all 15 test tubes with the help of acetate buffer. After the proper dilution absorbance was measured at 546 nm. Minimum 12 mg of given enzyme was required for the complete digestion of 10 mg substrate. Thus the quantity of enzyme required was 60% of the substrate quantity.

2.1.3. Papain:

The amount of papain required was based on the following principal. The quantity of papain required to digest casein completely in 24 hours at pH 5 (acetate buffer) and at 37 °C. It was prepared by dissolving 100 mg in 30 ml of acetic acid buffer (pH 5.0) and diluting to 100 ml with buffer, with final concentration 1 mg/ml. It was prepared by adding 200 mg of accurately weighed casein in sufficient warm water with constant stirring and diluted to 100 ml with final concentration 2 mg/ml. The

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solution was neutralized with 0.01M NaOH solutions using pH paper.

2. 1. 3.1. Method:
In 15 test tubes (numbered from 1 to 15), each containing 5 ml (i.e. 10 mg) of casein solution blank, 0.2,0.4,0.6,0.8,1.0,2,3,4,5,6,7,8,9 and 10ml of test solution of papain was added respectively. All the test tubes were placed in beaker, which was kept for shaking for 24 hours at room temperature. At the end of the period, the content of each test tube was titrated with 0.01 N NaOH using phenolphthalein as indicator. Minimum 6 mg of given enzyme was required for the complete digestion of 10 mg substrate. Thus the quantity of enzyme required was 60% of the substrate quantity. (6x100/10 = 60%).

2.1.4. Pepsin:
The quantity of pepsin required was determined by the same principal as that of papain i.e. the quantity of pepsin required to digest standard substrate completely in 24 hours at pH 5 and at 37°C. Similar method was used as that of papain. Minimum 6 mg of given enzyme was required for the complete digestion of 10 mg substrate. Thus the quantity of enzyme required was 60% of the substrate quantity.
(6x100/10 = 60%)

2.1.5. Lipase:
The quantity of lipase required was decided on the basis of the quantity of it required to hydrolyze standard substrate (olive oil) completely in 24 hours at pH 5 and at 37°C. It was prepared by dissolving accurately weighed 200 mg lipase in 60 ml of acetate buffer and volume was made to 100 ml with final concentration 2 mg/ml. Olive oil was used as standard substrate. 1.0 g of olive oil was weighed and dissolved in the mixture of methanol and diethyl ether (1:1) and diluted to 50 ml with final concentration 20 mg/ml.

2.1.5.1. Method:
In 15 test tubes (numbered from 1 to 15), each containing 1 ml (i.e. 20 mg) of standard substrate solution blank, 0.2,0.4,0.6,0.8,1.0,2,3,4,5,6,7,8,9 and 10 ml of test solution of lipase was added respectively. All test tubes were placed in a beaker and kept for shaking for 24 hours at 37°C. At the end of 24 hours, each test tube solution was titrated with 0.1M KOH solution using phenolphthalein as indicator. Minimum 12 mg of given enzyme was required for the complete hydrolysis of 10 mg substrate. Thus the quantity of enzyme required was 60% of the substrate quantity (12x100/20 = 60%). The dose of enzymes calculated for *Andrographis paniculata* are mentioned in Table 1.

Table 1. Enzyme quantity:

Sr.No.	Standard substrate	% Present in drug	Enzyme used	*Andrographis paniculata* powder (g)	Loading dose of enzymes (mg)
1.	Carbohydrate	20.18	Amylase	2.5	0.201
			Cellulase	2.5	0.302
2.	Protein	5.65	Papain	2.5	0.084
			Pepsin	2.5	0.084

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3.	Fat	1.6	Lipase	2.5	0.024

2.2. Screening of individual enzyme:

Screening of individual enzyme as well as screening of enzyme blend at 37 °C was processed in the following steps.

2.2.1. Pretreatment of crude drug with enzyme:
Quantity of crude drug:
The quantity of crude drug taken was 2.5 g (#80), which was dispersed in 50 ml of buffer media (acetic acid buffer pH 5). Throughout the study this quantity was kept constant. The effect of enzyme to facilitate drug release with respect to time was studied at 37°C, in a shaker kept at 135 rpm. Time interval of 1, 2, 4, 8, 12 and 24 h were fixed for the studies.

Experimental set up for the enzymatic pretreatment
Material and method:
The solution of various enzymes was prepared in acetic acid buffer I.P (pH =5.0). 0.201 g of amylase, 0.302 g of cellulase, 0.084 g of papain, 0.084 g of pepsin and 0.024 g of lipase were dissolved separately in sufficient acetate buffer and volume of each solution was made to 50 ml. 2.5 g of crude drug powder was suspended in 50 ml of buffer solution containing required quantity of enzyme. Six such conical flasks were prepared and kept for shaking on orbital shaker incubator at 37 °C for time intervals of 1, 2, 4, 8, 12 and 24 h respectively. After stipulated time intervals, suspension in each conical flask was filtered using vacuum. The filtrate was used for the analysis of sugar, protein and drug release, while the residue was dried in incubator and was used for the analysis of drug and fat release. At the same time control was carried out under identical condition replacing enzyme solution by 50 ml of buffer to compare the effect of enzyme.

2.2.2. Protein determination:
The Lowry method, which combines the use of the Biuret reaction of protein with copper ions in alkali with the reduction of Folin-Cio calteau phenol reagent by tyrosine and tryptophan residue.

2.2.3. Carbohydrate determination:
For the routine analysis of sugars DNS method was used.

2.2.4. Fatty acid determination:
For the routine purpose, titrimetric method was used using 0.05 M KOH solution using phenolphthalein indicator.

2.2.5. Determination of Drug Release:
To study the effect of enzyme on the facilitation of Andrographolide release, pretreated crude drug was analyzed by UV method. For this purpose every time drug released from fixed quantity of pretreated dried crude drug residue within fixed time was analyzed.

2.2.5.1. Method:
1.0 g of accurately weighed dried pretreated crude drug residue was extracted with 20 ml benzene, for 20 minutes by stirring at a constant speed on a magnetic stirrer. Then it was filtered. The residue was dried and mixed with sufficient kieselguhr and again extracted with 60 ml chloroform for 20 minutes. The extract was filtered; the filtrate obtained was concentrated and to this sufficient methanol was added to make the volume to 10 ml. From this 1 ml was taken and diluted to 25 ml and absorbance was measured at 226 nm with reference to blank. The concentration was calculated with the help of

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standard curve described in standardization of UV method of andrographolide analysis.

2.3. Design and screening of enzyme blend:

For 2.5 g of crude drug, the various individual enzymes was used in following doses:

Amylase: 0.201 g; Cellulase 0.302 g; Papain: 0.084 g; Pepsin: 0.084 g; Lipase: 0.024 g.

For 2.5 g of crude drug, the blend was prepared by adding and mixing all above mentioned enzymes in respective doses i.e. in the ratio of 4:6:2:2:1 in sufficient quantity of acetic acid buffer and volume was made to 100 ml. From the above-prepared blend quantity equivalent to 10 % and 20 % w/w of 2.5 g of crude drug was used for the enzymatic pretreatment, which is summarized in Table 2.

Table 2.

Blend ratio of crude drug

% Blend	Wt. (mg) of blend	Corresponding vol. (ml) of blend
10%	250	17.98
20%	500	35097

RESULT AND DISCUSSION

The enzymatic pretreatment of *Andrographis paniculata* was studied to facilitate the extraction of andrographolide. The screening of individual enzyme was done by analyzing sugar, protein, drug and fat release. The work was also useful to study the behavior of enzyme towards crude drug. It was found that sugar release was not only restricted to amylase and cellulase, but lipase, papain and pepsin were also showing sugar release. At the same time protein release was not restricted to papain and pepsin but lipase, amylase and cellulase were showing protein release. This behavior of enzyme gives support to the hypothesis that polysaccharides and polypeptides polymer are interlinked with each other to form complex network. It is important to note that the *Andrographis paniculata* contains 20.18 % of the carbohydrate (starch). The enzyme, which acts on it i.e. on carbohydrate, can decrease the amount of the starch; and hence intern decreases the preload of the starch for extraction of andrographolide. The action of carbohydrate is not restricted only to starch but it acts on protein also. Hence it proves that the andrographolide is present in the matrix of protein and polysaccharides. Papain and pepsin acts on the protein and decrease the extraction load, which intern help in extraction. These two enzyme acts on the protein as well as on the starch. The ease in the andrographolide extraction by different enzymes shows that cellulase is most useful and their usefulness is in order of: Cellulase> Amylase > Blend 20 % > Pepsin > Papain >Blend 10 %>lipase. Cellulase shows more extraction of sugar and drug release, while papain showed maximum protein release. The designed enzyme blend 20 % also shows better result as compare to other enzymes. In case of blank (pretreatment without enzymes), it was found that sugar, protein, drug and fat release were almost constant. The sugar release by different enzyme and their enzyme blend was in the following order: Cellulase>Amylase>Blend 20 %>Papain>Pepsin>Blend 10 %>Lipase. It was observed that enzyme which releases more amount of sugar also releases more amount of drug because of the action of the starch. The protein release by different enzyme and their enzyme blend was in the

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following order Papain>Pepsin>Blend 20 %>Cellulase>Amylase > Lipase>Blend 10 %. The drug release by different enzyme and their enzyme blend was in the following order: Cellulase>Amylase>Blend 20 %>Papain>Pepsin>Lipase>Blend 10 %. The enzyme pretreatment *of Andrographis paniculata* gives more extraction of andrographolide but still this study also requires some optimization like, optimization of temperature, speed of shaker, pH of buffer media etc.The results obtained from comparative study of enzymatic activity are mentioned in Table 3.

Table 3. ***Comparative Study of Enzymatic Activity***

Time interval (h)	% Release	Blank	Amylase	Cellulase	Papain	Pepsin	Lipase	Blend 10% w/w	Blend 20% w/w
1 hr.	C	5.65	7.40	9.70	7.32	7.25	6.90	5.81	7.44
	P	0.053	1.31	1.46	1.58	1.57	1.23	1.26	1.36
	D	0.003	0.0246	0.049	0.0214	0.0285	0.018	0.036	0.0862
	F	0.23	0.23	0.35	0.23	0.23	0.35	0.23	0.23
2 hr.	C	5.67	8.4831	9.95	7.64	7.55	7.27	5.99	9.31
	P	0.055	1.56	1.57	2.13	2.10	1.54	1.93	1.72
	D	0.077	0.044	0.071	0.049	0.0640	0.028	0.0613	0.0914
	F	0.23	0.35	0.47	0.35	0.35	0.47	0.35	0.35
4 hr.	C	5.70	10.07	10.65	8.27	7.79	7.67	6.50	10.69
	P	0.062	2.10	2.23	2.65	2.30	1.76	2.17	2.41
	D	0.009	0.070	0.15	0.079	0.092	0.059	0.0967	0.176
	F	0.23	0.47	0.47	0.35	0.47	0.71	0.35	0.47
8 hr.	C	5.72	10.28	11.50	8.92	8.26	7.97	6.73	12.13
	P	0.074	2.17	3.16	3.34	3.14	2.43	2.51	3.06
	D	0.013	0.141	0.150	0.129	0.1116	0.071	0.131	0.217

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	F	0.23	0.83	0.71	0.47	0.47	0.95	0.37	0.59
12 hr.	C	5.97	11.89	13.89	9.12	8.65	8.76	7.96	12.83
	P	0.091	3.12	3.74	3.95	3.85	2.90	2.93	3.87
	D	0.0242	0.23	0.33	0.23	0.186	0.092	0.167	0.296
	F	0.23	0.95	0.83	0.47	0.59	1.07	0.37	0.59
24 hr.	C	6.15	13.78	15.36	10.56	12.91	9.20	9.27	13.70
	P	0.124	3.48	4.00	4.43	4.28	3.28	3.16	4.18
	D	0.036	0.34	0.37	0.31	0.262	0.101	0.201	0.34
	F	0.23	1.07	1.07	0.59	0.71	1.31	0.49	0.71

C: Carbohydrate, P: Protein, D: Drug, F: Fat

CONCLUSION

The study of enzymatic treatment to facilitate andrographolide release was studied and amongst the various enzymes tried cellulase was found to be the most effective in improving the extraction yield of andrographolide. The above work highlights the enzymatic pretreatment of the crude drug powder, which leads to ease in the extraction of the phytoconstituent of interest.

REFERENCES

1. S.S.Handa, Anupam sharma, Hepatoprotective activity of Andrographolide from Andrographis paniculata Nees, Indian J.Med.Res [B], 92:276-283, (1990).
2. K.Senthil Kumaran, P.Thirugnanasambantham, S. Viswanathan, M. Sree Rama Murthy. An HPLC method for the estimation of Andrographolide in Rabbit serum, Indian Journal of Pharmacology, 35:109-112, (2003).
3. S. Saxena, D.C.Jain, R.S.Bhakuni, R.P. Sharma.. Chemistry and Pharmacology of Andrographis species, Indian Drugs, 35: 458-466, (1998).
4. M. George, K.M. Pandalai. Investigation on plant antibiotics. Part IV. Further search for antibiotic substances in Indian medicinal plants. Indian Journal of Medical Research, 37:169-181, (1949).
5. K. Nakanishi. Phytochemical survey of Malaysian plants: preliminary chemical and pharmacological screening, Chemical and Pharmaceutical Bulletin, 13: 82-890, (1965).
6. A. .Leelarasamee. Undetectable antibacterial activity of *Andrographis paniculata* (Burm) Wall.ex Nees. of the Medical Association of Thailand ,73:299-304, (1990) Journal
7. S.K. Nazimudeen, Effect of *Andrographis paniculata* on snake venom induced death and its mechanism, Indian Journal of Pharmaceutical Sciences, 40:132-134, (1978).