ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

PHARMACOGNOSY

ENZYMATIC PRETREATMENT TO FACILITATE EXTRACTION OF ISOFLAVONES FROM *SOJA HISPIDA*.

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ABSTRACT

Soyabean is widely utilized nutraceutical used for addressing conditions like post menopausal syndrome and antioxidant properties. It is thought that the active principles are present in a proteinaceous and polysaccharide matrix. Hence, the objective of the present work was to study the effect of enzymatic pretreatment of Soyabean to facilitate the extraction of isoflavones. Pepsin and papain enabled 0.194 and 0.188 % extraction of total isoflavones from Soya bean. Pepsin was found to be most effective in releasing isoflavones as compared to other ENZYMES AND ENZYME BLEND.

KEYWORDS

Soja Hispida, Enzymes, Extraction, Isoflavones

INTRODUCTION

Plant extracts and extracts enriched with phytoconstituent besides being used in healthcare systems are also being increasingly used in nutraceutical products. The recent trend is to use herbal drugs as nutraceuticals. Nutraceuticals are chemicals found as a natural component of foods or other ingestible forms that have been determined to be beneficial to the human body in preventing or treating one or more diseases or improving physiological performance.[1]

Nutraceutical products are increasingly being used for addressing conditions like obesity, arthritis, diabetes, postmenopausal syndrome, osteoporosis, antioxidant, etc.[2] Soyabean phytoconstituents have been found to be an integral part of nutraceuticals. Soyabean contains isoflavones which behave as phytoestrogens. Phytoestrogen is the term applied to non-steroidal plant materials displaying estrogenic properties. Isoflavones are planar molecules, which mimic the shape and polarity of the steroid hormone estradiol and are able to bind to an estrogen receptor, though their activity is less than that of estradiol. In some tissues, they stimulate an estrogenic response, whilst in others they can antagonize the effect of estrogens. Such materials taken as part of the diet therefore influence overall estrogenic activity in the body by adding their effects to the normal levels of steroidal estrogens .[3]

Soybean contains isoflavones, namely: Genistin, Daidzin, Glycitin, Glycitein 7β-O-glucoside and their aglycones Genistein, Daidzein and Glycitein respectively. The total isoflavone content varies from 0.1-0.4 %.[3, 4]

Since the active principles are present in a proteinaceous and polysaccharide matrix, if the matrix is broken down by some method, it may facilitate the extraction of active principles, thus saving time and energy. Conventional methods of extraction like usage of heat is less capable of breaking the cell matrix; while in chemical treatment, the polysaccharide matrix can be broken by acid hydrolysis, but there are chances of degradation of active constituents. Enzymatic pretreatment to facilitate cell matrix destruction can be a better method of choice due to non requirement of heat, less energy consumption, specificity of enzymes and milder conditions.[5]

Enzymatic pretreatment has been found to beneficial in the extraction of andrographolide fron *Andrographis paniculata*.[6] Therefore the objective of the present work was to study the effect of treatment of enzymes on Soyabean to facilitate the extraction of isoflavones.

MATERIALS AND METHODS

1.1.Standardization and Dose Optimization of Enzymes:

1.1.1. Amylase[7]:

The required amount of the amylase was determined by the ability of the enzyme to hydrolyze starch. 100 mg of given amylase sample was accurately weighed and dissolved in sufficient acetate buffer (pH 5) and volume was made to 100 ml. 200 mg corn starch was accurately weighed, added in sufficient distilled water and warmed gently and volume was made to 100 ml with water.

1.1.1.2. Method

Into each 15 test tubes (numbered from 1 to 15), 5 ml (i.e. 10 mg) of standard substrate solution was added and test tubes were placed in water bath maintained at 40 ^{0}C. When temperature of solution in the tubes had reached 40 ^{0}C, blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of test solution was added in no. 1 to 15 test tubes respectively. The

tubes were mixed thoroughly and kept in the water bath at 40 ^{0}C. After exactly an hour the tubes were removed and in each tube 0.05 ml of 0.02 M iodine was added, mixed and observed for the colour change. A minimum 4.0 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.

1.1.2. Cellulase[8, 9]:

The quantity determination is based on the principal that cellulase hydrolyses carboxymethylcellulose (CMC) and produces carboxymethyl oligosaccharides; which on reacting with 3, 5-dinitrosalisilic acid (3, 5-DNSA) produced red color which was measured at 546 nm. 500 mg (NaCMC) was dissolved in 100 ml acetate buffer (5 mg/ml). 200 mg of cellulase was weighed and added into the acetate buffer and the final volume was made to 100 ml with acetate buffer.

1.1.2.1. Method:

4 ml of Sodium carboxymethylcellulose was added to 15 test tubes (numbered 1-15). Precaution was taken that the solution should not adhere to the sides of test tube. In these test tubes blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of enzyme solution was added respectively. The tubes were put in orbital shaker incubator (37 ^{0}C, rpm = 135), for 24 hr. 1 ml DNS reagent was added. Volume was made upto 15 ml with acetate buffer. Absorbance was measured at 546 nm. A minimum 12 mg of dose of enzyme was required for complete digestion of 20 mg substrate. Thus the quantity of enzyme required was 60 % of the substrate quantity.

1.1.3. Papain[10]:

The quantity determination is based on the ability of papain required to digest standard substrate (i.e. casein) completely in 24 hr in acetate buffer at 37 ^{0}C. 100 mg of accurately weighed papain was dissolved in sufficient amount of acetate buffer and diluted to 100 ml. The subtstrate casein solution was prepared by dissolving 200 mg of casein in sufficient warm water and diluted to 100 ml. The solution was neutralized by 1 N NaOH (0.2 ml).

1.1.3.1. Method:

In 15 test tubes (numbered 1-15), 5 ml of casein solution (2 mg/ml) was added. Later blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of enzyme was added respectively in each test tube. All the test tubes were kept in beaker for shaking for 24 hr at 37 ^{0}C. After 24 hr, each test tube was titrated against 0.1 N NaOH using phenolphthalein as indicator. A minimum of 6 mg dose of papain was required for complete digestion of 10 mg of substrate. Thus the quantity of enzyme required was 60 % of the substrate quantity.

1.1.4. Pepsin[10, 11]:

The quantity of pepsin required was determined by the ability of the enzyme to digest standard substrate (casein) completely in 24 hr in acetate buffer at 37 ^{0}C. The test solution of pepsin was prepared by dissolving the accurately weighed 100 mg of enzyme in sufficient amount of acetate buffer and diluting to 100 ml with buffer. The substrate was prepared by adding 200 mg of casein in sufficient warm water and diluting to 100 ml. The solution was neutralized with 1N NaOH (0.2 ml).

1.1.4.1. Method:

In 15 test tubes (numbered 1-15), 5 ml of casein solution (2 mg/ml) was added. Later blank, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of enzyme was added. All the test tubes were kept in beaker for shaking for 24 hr at 37 ^{0}C. After 24 hr, each test tube was titrated against 0.1 N NaOH using phenolphthalein as indicator. A minimum 6 mg dose of pepsin was required for complete digestion of 10mg of substrate. Thus the quantity of enzyme required was 60 % of the substrate quantity.

1.1.5. Lipase[12]:

The quantity of lipase was decided on the basis of the quantity of enzyme required to digest standard substrate (Olive oil) completely in 24 hr at pH 5 and at 37 ^{0}C. The test solution of lipase was prepared by triturating accurately weighed 200 mg lipase in 60 ml of acetate buffer. The volume was made to 100 ml with buffer. Olive oil is used as standard substrate. 1 g of accurately weighed olive oil was dissolved in methanol and diethyl ether (1:1) and diluted to 50.0ml.

1.1.5.1. Method:

In 15 test tubes (numbered from 1 to 15), each containing 1.0 ml (20 mg/ml) 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, which in turn was kept for shaking for 24 hr at 37 ^{0}C. After 24 hr, the solution of each test tube was titrated with 0.1 M KOH solution using phenolphthalein as indicator. A minimum of 12 mg of enzyme would be required for complete digestion of 20 mg of substrate. Thus the quantity of enzyme required was 60 % of the substrate quantity.

Table 1 describes the calculated amounts of carbohydrates, proteins, fats present in soyabean and the required dose of individual enzymes for its pretreatment.

Table 1.
Calculated dose of enzyme for pretreatment

Sr.No	Standard substrate	% Present in drug	Enzymes used	Soyabean powder (g)	Loading dose for enzymes (mg)
1	Carbohydrates	33%	Amylase Cellulase	3	396 594
2	Proteins	37%	Papain Pepsin	3	666 666
3	Fats	19%	Lipase	3	342

1.2. Screening Of Individual Enzymes:

Screening of individual enzymes at 37 ^{0}C as well as screening of enzymatic blend was processed in the following steps:

1.2.1. Pretreatment of crude drug with enzyme:

Quantity of crude drug: 3 g crude drug (80 mesh) was used every time dispersed in 50 ml of buffer medium (pH 5). Throught this study, the quantity of the drug was kept constant. The enzyme mediated phytoconstituents release with respect to time was carried out at 37 ^{0}C in a shaker kept at 135 rpm. Time interval of 1, 2, 4, 8, 12 and 24 hr were fixed.

Experimental set up for the enzymatic pretreatment:

MATERIAL AND METHOD

The enzyme solutions were prepared in acetic acid buffer I.P (pH 5). 0.396 g dose of amylase, 0.594 g dose of cellulase, 0.666 g dose of papain and 0.666 g dose of pepsin, 0.342 g dose of lipase were dissolved separately in sufficient acetate buffer and the volume of each solution was made to 50 ml. 3 g powdered crude drug was suspended in a 50 ml of enzyme containing buffer solution (containing required dose). Such 6 conical flasks were prepared and kept for shaking on orbital shaker incubator at 135 rpm maintained at 37 ^{0}C for interval 1, 2, 4, 8, 12 and 24 hr. After the period of respective time interval, the conical flask were removed and

filtered on vacuum pump. The filtrate was analyzed for sugar and protein release, while the residue was dried in the incubator and used for analysis of fat and drug release. At the same time blank was carried out under identical conditions replacing enzyme solution by 50 ml buffer to compare the effect of enzymes.

1.2.2. Sugar determination[13]:

Analysis of sugars was done by using 3, 5-Dinitrosalicylic acid (DNS) method.

1.2.3. Protein determination[14]:

Protein release was analysed by Folin-Ciocalteau reagent

1.2.4. Fat determination[15]:

Fat release was determined using titrimetric method with 0.05 M KOH solution using phenolphthalein indicator.

1.2.5. Determination of drug release[16]:

To study the effect of enzymes on the facilitation of total isoflavones release, pretreated crude drug residue was analyzed by colorimetric method. For this purpose every time, drug released from fixed quantity of pretreated dried residue within fixed time was analyzed.

1.2.5.1. Method:

1 g of accurately weighed dried pretreated crude drug residue was extracted with 50 ml of 80 % methanol for 20 min by magnetic stirring. Thereafter the extract was filtered into a 50 ml volumetric flask. Volume was made with 80 % methanol. 2 ml aliquot was withdrawn and evaporated to 1 ml and hydrolyzed by 5 ml of 6 % HCl. The hydrolysis was conducted on a boiling water bath for 45 min. The solution was cooled and the free aglycones were extracted with 4 ml of solvent ether. The ether was removed and evaporated. The residue was dissolved in 2 ml of methanol. The methanolic solution was analyzed for the total isoflavones by colorimetric method using Folin-Ciocalteau reagent (50 %), and read the absorbance at 750 nm after 30 min.

1.3. Design and Screening Of Enzyme Blend:

For 3 g of crude drug, various individual enzymes were used in following doses: amylase: 0.396 g, Cellulase: 0.594 g, Papain: 0.666 g, Pepsin: 0.666 g, and Lipase: 0.342 g.

For 3 g of crude drug, the blend was prepared by adding and mixing all above-mentioned enzymes in respective doses in sufficient quantity of acetic acid buffer pH 5 and volume was made to 100 ml. From the above-prepared blend, quantity equivalent to 10 % and 20 % w/w of 3 g of crude drug was used for the enzymatic pretreatment, which is summarized in Table 2.

Table 2
Blend ratio of enzymes

% Blend w/w	Wt. in mg of blend	Corresponding vol. (ml) of blend
10%	300 mg	11.2 ml
20%	600 mg	22.4 ml

RESULT AND DISCUSSION

The enzymatic pretreatment of *Soja hispida* was studied to facilitate the extraction of isoflavones. Although the study was elementary in nature but it shows that enzyme can be effective to facilitate extraction of active constituents with saving energy consumption and improved yield. The effect of individual enzyme was studied on the release of sugar, protein, drug and fat

from polymetric matrix. 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. The ease in the isoflavone extraction by different enzymes shows that pepsin is most useful and their usefulness is in order of: Pepsin > Papain > Amylase > Blend 20 % > Cellulase > Lipase > Blend 10 %. The sugar release by different enzyme and their enzyme blend was in the following order: Pepsin > Papain > Blend 20 % > Amylase > Blend10 % > Cellulase > lipase. It was observed that enzyme which releases more amount of sugar and protein also releases more amount of drug. The protein release by different enzyme and their enzyme blend was in the following order: Pepsin > Papain > Amylase > Cellulase > Blend 20 % > Lipase > Blend 10 %. Considering the composition of soyabean, the above result is justified. The fat release by different enzymes and their blend was in the following order: Lipase > Blend 20 % > Blend 10 % > Pepsin > Papain > Amylase > Cellulase. The results from the comparative study of enzyme facilitated release are tabulated in Table 3.

Table 3.
Comparative Study of Enzymatic Activity

Time Interval	% Release	Blank	Amylase	Cellulase	Papain	Pepsin	Lipase	Blend 10%w/w	Blend 20%w/w
1hr	S	2.78	10.76	4.46	18.3	15.66	8.43	7.8	14.9
	P	3.32	15.34	19.86	16.20	15.49	10.23	6.97	13.21
	L	0.7	1.43	1.19	1.43	1.19	3.59	3.11	3.83
	D	0.0036	0.0451	0.022	0.062	0.078	0.0272	0.0207	0.0531
2hr	S	2.92	13.17	5.20	20.7	20.11	10.97	10.53	16.97
	P	3.69	17.81	19.95	17.07	18.21	10.91	7.67	13.89
	L	0.95	1.67	1.67	1.80	1.80	4.78	3.59	4.78
	D	0.0087	0.0625	0.0314	0.079	0.094	0.028	0.0381	0.0745
4hr	S	3.05	14.47	7.23	23.3	23.6	12.75	12.2	17.9
	P	3.97	20.05	20.00	19.41	19.98	11.56	10.53	15.37
	L	1.43	1.80	2.39	2.39	2.39	6.22	4.30	6.22
	D	0.0124	0.071	0.0404	0.102	0.115	0.038	0.0418	0.0797
8hr	S	3.55	15.23	7.36	27.5	28.2	13.0	13.1	19.1
	P	4.95	22.08	20.43	21.61	23.46	14.36	12.24	16.98
	L	1.67	3.11	2.87	3.59	2.87	7.18	4.78	7.66
	D	0.0184	0.079	0.0498	0.121	0.131	0.0481	0.0491	0.0813
12hr	S	4.22	17.42	8.69	28.8	29.2	13.32	14.05	20.3
	P	5.06	23.29	22.29	22.4	24.29	16.54	14.41	18.25
	L	1.80	3.59	3.35	4.06	3.59	11.97	6.70	9.09
	D	0.0211	0.0842	0.0551	0.146	0.153	0.0525	0.0518	0.0857
24hr	S	4.47	20.1	15.8	29.49	30.9	14.23	16.29	21.25
	P	5.42	24.66	24.61	25.25	29.83	20.51	16.65	24.57
	L	2.39	4.06	3.83	4.30	5.02	13.17	7.18	10.05
	D	0.0258	0.0973	0.0575	0.188	0.194	0.0565	0.0545	0.0957

S: Average % sugar release, P: Average % protein release, L: Average % fat release, D: Average % drug release.

CONCLUSION

The enzymatic pretreatment of *Soja hispida* was studied to facilitate the extraction of isoflavones. Enzymatic pretreatment gave impoved extraction of isoflavones than the blank. The study shows that enzyme can be effective to facilitate extraction of active constituent with saving energy consumption and improved yield. The work was also useful to study the behavior of enzyme towards drug. The above works highlights the importance of enzymatic pretreatment of the crude drug powder, which can lead to an ease in the extraction of the phytoconstituent of interest.

ACKNOWLEDGEMENT

R.R.B and R.S.G are grateful to the University Grants Commission (UGC) for providing Junior Research Fellowship for this project.

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