CHAPTER 3

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CHAPTER 3

RESULTS & DISCUSSION

Amylose is a straight chain component of starch. Under certain conditions it is known to undergo conformational changes allowing selective adsorption of certain molecules. Starch has been used to effect adsorption in separation of amino acids. The complex forming ability of amylose and its use in isolation of l-arginine- HCl has been developed. The method has been extended to purify a guanidino compound from alfalfa plant - a highly valued fodder used for domestic animals (herbivorous).

The present account is an attempt in this direction and affords a convenient, easy, elegant and reproducible method for the isolation of l-arginine-HCl and low molecular weight guanido compounds present in natural juice or extracts.

3.1 ADSORPTION OF POLAR MATERIAL BY STARCH

The adsorptive property of starch paste and solution is due to its component amylose. The starch

solution when treated with polar organic substances having a hydrophilic group (e.g. hydroxyl or carboxyl) attached to hydrophobic residue. The adsorption of it is caused by amylose due to polar attraction for hydrophilic group. The resulting adsorption complex is insoluble and consequently separates from the solution.

The isolated amylose forms intense blue coloured complex with iodine while amylo pectine gives black to purple complex. The amylose is responsible for the adsorption of various polar material. This property has been utilised to effect quantitative precipitation of amylose. A selective precipitation of amylose gel was accomplished by treating a starch paste with 8 percent n-butanol, constantly heating for 3 hours at 80° C. On cooling to room temperature and subsequent refrigeration, the amylose fraction separated in gel form could be collected by centrifugation. The yield was 22 - 23 percent from potato starch. The reported value of iodine adsorption of the crude precipitated amylose was 16.5 percent; for the non-precipitated amylopectin fraction, it was 1.5 to 1.7 percent.

Most of the monohydroxy alcohols will accomplish a separation under suitable conditions which include n-amyl alcohol, pentasol, n-propyl alcohol, n-hexyl alcohol, 2-ethyl-1-butanol, 2-ethyl-1-hexanol, lauryl alcohol, cyclohexanol, n-butanol, isoamyl alcohol, benzyl alcohol, etc. Of these, pentasol gives optimum results while n-butanol also gives good results.

The degree of granule swelling in gel is increased by the use of lower aliphatic alcohols. The gelatinization temperature is also lowered. Reverse effect has been observed at higher alcohol concentration. Example, higher alcohol like lauryl alcohol or cyclohexanol restrict the granule swelling. Hence n-butanol is probably adsorbed via its hydroxyl group, the alcoholate then presents a hydrophobic surface to the medium and precipitates from solution. Amylose, due to its extended length and high content of hydrophilic group, sets up a zone of attractive influence for hydrophilic substances and causes aggregation of the particles, hence colloidal dimension is resulted, precipitating from solution. This process is known as Retrogradation. It is a relatively, slow process occuring over a period of hours or even days.

3.2 ANALYSIS OF ALFALFA FILTRATE

The extract of alfalfa leaves and soft stem in distilled water, was having a pH of 8.5. This indicated the possibility of a large content of basic amino acids. The analysis of the alfalfa filtrate gave the following results :

1) The free amino concentration of alfalfa filtrate determined by ninhydrin reagent was 15 percent.
2) The protein concentration by Lowry's Method before dialysis was 16 percent.
3) The total carbohydrate content, determined by phenol sulphuric acid method was 5 percent.
4) The concentration of arginine determined by Sakaguchi reaction was 6 percent.

(Quantities expressed in gm/100 gms of concentrated extract.)

3.3 ADSORPTION OF ALFALFA EXTRACT ON AMYLOSE GEL

The separation of basic amino acids, especially, guanidine was quantitatively carried out using amylose gel. This procedure was rapid and easy to carry out. Our

interest was more with guanidino amino acids hence this procedure was useful. The adsorption of alfalfa filtrate was carried out at various conditions to optimise adsorption of basic amino acids on amylose.

1) Adsorption at various pH :- The amylose gel (150 ml) was mixed with alfalfa extract (150 ml containing 4 mg of amino acids), at varying pH. The result of adsorption are reported in Table XI. (Fig. IX, X)

ADSORPTION OF LUCERNE EXTRACT ON AMYLOSE GEL

SR.NO.	pH	ADSORPTION %	DESORPTION %
1	3.5	35	20
2	4.0	25	16
3	4.5	60	42
4	5.0	35	30
5	6.5	16	8

The adsorption was found to be more effective at pH 4.5 and 2.6. For preparative work in the present investigation, the adsorption was carried out at pH 4.6. The process of adsorption with citrate buffer at pH 4.6

and desorption with phosphate buffer at pH 7.5 under similar conditions was carried out. The result is reported in Table XII.

TABLE XII

ADSORPTION & DESORPTION OF ALFALFA EXTRACT ON AMYLOSE GEL IN DIFFERENT BUFFERS

SR.NO.	BUFFER	ADSORPTION %	DESORPTION %
1	Citrate	60	50
2	Phosphate	40	20

When amylose gel was treated with 70 percent alcohol under similar conditions of pH and ionic strength, the following results in Table XIII were obtained.

TABLE XIII

ADSORPTION & DESORPTION OF ALFALFA EXTRACT ON AMYLOSE GEL WITH 70 % ALCOHOLIC TREATMENT

SR.NO	ADSORPTION %	DESORPTION %
1	65	50
2	45	20

The results obtained showed 70 percent alcohol concentrate favoured the maximum adsorption of basic amino acids on amylose gel. The process was carried out with citrate buffer at pH 4.5 - 4.6. The recovery of guanidine compound (including arginine) by this method was 90 percent. The alfalfa filtrate containing 6 gms of amino acids provided about 900 mg of guanidine positive content by this method.

3.4 PAPER CHROMATOGRAPHY

The sample obtained by the process of adsorption and desorption was subjected to paper chromatography. The clear ninhydrin positive spot of arginine and unknown compound along with traces of lysine, aspargine, tyrosine, aspartic acid and glutamic acid were obtained. The Rf values of amino acids are reported in Table XIV. (Fig. XI)

TABLE XIV

Rf VALUES OF AMINO ACIDS DESORBED ALFALFA EXTRACT FROM AMYLOSE GEL

SR.NO.	AMINO ACID	Rf	SR.NO.	AMINO ACID	Rf
1	Arginine	0.11	4	Glutamic acid	0.16
2	Unknown	0.20	5	Aspartic acid	0.13
3	Tyrosine	0.32	6	Lysine	0.10

3.5 ION-EXCHANGE CHROMATOGRAPHY

The chromatographic column of Dowex 50 X 4 (30 x 1.2 cms) was used for the ion-exchange chromatography The separation of amino acid on an ion-exchange column results from ionic and hydrophobic interactions between the amino acid molecule and resin. The degree of resolution achieved is dependent on these interactions and on the flow rate of eluting buffers through the column. The amino acids eluted are generally in the group order of acidic, neutral and basic amino acids on cation exchange resin. This elution order arises from pKa values of amino acids (119), branching and shortness of nonpolar side chains decrease elution time (120).

As the strength and pH of the eluting buffer was increasing in 0.2 M pyridine acetate buffer of pH 4.5, the guanidine positive peak was obtained at pH 6. The results obtained with original alfalfa filtrate on cation exchanger Dowex 50 X 4, under similar conditions showed the presence of aspartic acid, aspargine, glutamine, serine, alanine and leucine. The recovery of arginine positive compound was 95 percent. The diagramatic representation of ion-exchange chromatography of alfalfa filtrate is shown in Fig.XII.

3.6 GEL FILTRATION CHROMATOGRAPHY

The arginine positive fraction obtained from the ion-exchange chromatography was further subjected to gel filteration. The elution pattern of Bio-gel P-2, loaded with 2 mg of guanidine sample showed highest concentration at the fraction numbers 15,16, and 17. These results are represented in Fig.XIII. These three fraction of 1 ml were pooled and concentrated and subjected to rechromatography on the same column showed highest concentration in the fraction number 15, with a void volume of 10 ml. (Fig XIV)

The fraction number 15 from the second lot of Bio-gel P-2 chromatography was reloaded once again on the same column, the result of which showed the highest activity in the fraction number 14 with void volume of 9 ml. (Fig. XV)

The carbohydrate content increases with increase in the amino acid content and with the increase in concentration of guanidine compound.

Paper Chromatography :- The purified guanidine compound which may be amino sugar gave the Rf value of 0.2, using the solvent system of butanol-acetic acid-water in ascending chromatography.

3.7 ENZYME TREATMENT

The semi-purified arginase enzyme was highly active with arginine as a substrate, activity was free of protease and carbohydratase. The specificity of the enzyme was tested using amino acids other than arginine e.g. tyrosine, which was not degraded by the enzyme.

The optimum catalytic activity of arginase (sheep liver) was found when the assay was performed at pH 9.5 using glycine buffer. The optimum concentration of arginine was 20 mM per liter and in presence of $MnCl_2$ (5 mM) as an activator at 37° C. The result of the enzyme assay is shown in the Table XV.

TABLE XV

ENZYME ASSAY RESULTS

ASSAY	ARGININE CONC. ON 1 HR. INCUBATION	ARGININE CONC. ON OVERNIGHT INCUBATION
0.1 ml of arginine + 6 ml H_2O + 0.1 ml buffer + 0.1 ml Enzyme + 0.1 ml $MnCl_2$	0.85 mM	1 mM
Arginine fraction 0.1 ml + 0.6 ml H_2O + 0.1 ml buffer + 0.1 ml Enzyme + 0.1 ml $MnCl_2$	0.85 mM	0.85 mM

The arginine positive fraction when treated with arginase enzyme under similar conditions, increasing the incubation period upto 48 hours. The reaction system was treated with ·5 percent perchloric acid to denature the enzyme protein. The precipitate formed was removed by centrifugation at 10,000 g. The supernatent was concentrated and utilised to separate amino acids on the Dowex cation exchange column.

The amino acids were eluted using the conditions of Moore and Stein (110) as described earlier. The guanidine positive fraction at pH 6 was identified by Sakaguchi Reaction. (Fig XVI)

3.8 ARGINASE SPECIFICITY

Arginase isolated from various sources (123) shows a high degree of specificity. The enzyme hydrolyses l-arginine into l-ornithine and urea. The molecules containing guanidino group viz. agmatine, d-arginine, -guanidinobutyric acid, B-guanidopropionic acid, trypsin digested bovine serum albumin, l-arginine AH-sepharose 4B, and streptomycin do not undergo hydrolysis. Arginase requires α -amino acid and α -carboxylic group free probably for binding of the substrate molecule to the

binding locus of the active site of arginase as a result of which the suceptible bonds of l-arginine $-\overset{\overset{O}{\|}}{C}\sim NH-CH_2$ come in close proximity of the catalytic site to undergo hydrolysis liberating urea as one of the products. $-\overset{\overset{O}{\|}}{C}\sim NH-CH_2$ linkage in l-arginine is the absolute requirement of the enzymic action. Simple binding through α $-NH_2$ and α -COOH groups to the binding locus of the enzyme is not enough, but guanidino group of the substrate must fit in the right position in the cavity that serves as a catalytic centre. Thus arginase enzyme is very very specific and hydrolyses only l-arginine. In a natural extract quantitative analysis of free l-arginine is simple. The chemical nature of the amino acid side chains specifically containing guanidino groups could be differentiated from l-arginine depending upon its response to hydrolytic action of arginase.

The guanidino group, positive fraction isolated from alfalfa extract on treatment with arginase under optimal catalytic conditions, did not liberate urea as one of its product. The results are indicative of the following : (Fig XVII)

1. The prescence of unusual amino acid containing guanidino group.

2. The α-NH_3 and /or α-COOH of l-arginine is modified,
3. It may be D-arginine,
4. The guanidino group modification is remote due to its response towards Sakaguchi Reaction.

Our observation supports the first two possibilities. The fraction was subjected to extensive purification on ion-exchange and gel filteration chromatography. The molecular sieving media, Bio-Gel P_2 is used to seperate molecules having molecular size in the range of 100 - 1800 daltons. The molecular weight of l-arginine being 176 must therefore, elute in the proximity of total elution volume. The position of the compound of interest however, is very close to void volume indicative of a higher molecular sized component.

Hence, this guanidino compound is having molecular weight in the range of 1500 - 1800 dalton showing its oligopeptide nature. The complete hydrolysis of oligopeptide under present study shows higher content of arginine, as the intense spot was recorded on the paper chromatogram. Further, it is eluted at the alkaline pH from the Dowex 50 X 4, confirming its basic nature. Since the compound is not acted upon by the enzyme arginase, its

N - terminal or C - terminal may be modified or glycosylated. This conclusion is supported by the sugar and amino sugar positive reactions.

Thus, the guanidino compounds isolated from the alfalfa which is free in nature, may be an oligopeptide having a high content of arginine. It may be the degradative product of high molecular weight protein. The arginine rich compound may be of biological importance.