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SEPARATION AND CHARACTERIZATION OF TWO FORMS OF ACETOHYDROXY ACID SYNTHASE FROM BLACK MEXICAN SWEET CORN CELLS

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SUMMARY

Acetohydroxy acid synthase (EC 4.1.3.18), the first enzyme unique to the biosynthesis of the branched chain amino acids valine, leucine and isoleucine, is the site of action of the two different classes of herbicides, the imidazolinones and the sulfonylureas. Multiple forms of this enzyme with different sensitivity to feedback inhibition by amino acids or to inhibition by herbicides have been reported in microorganisms only, although the presence of similar forms of isozymes in plants has long been speculated. This is the first report of the isolation of two forms of acetohydroxy acid synthase from a plant source. The two forms were separated by both fast protein liquid chromatography and conventional chromatography. These forms exhibit significant differences in their physical and kinetic properties as well as in their sensitivity to inhibition by amino acids and by different herbicides.

INTRODUCTION

Acetohydroxy acid synthase (AHAS; also known as acetolactate synthase) is a key controlling point for the levels of the branched chain amino acids in both prokaryotes and eukaryotes. In bacteria, this control involves feedback inhibition of enzyme activity by the amino acids or repression of enzyme synthesis or both[1]. In plants, however, the only known mechanism of the regulation of this enzyme is through feedback inhibition by valine, leucine and isoleucine[2,3]. In spite of its important role, eukaryotic AHAS has been studied to a very limited extent, probably because of its labile nature[4–7].

AHAS has received special attention in recent years since the findings that two different new classes of herbicides, the imidazolinones and the sulfonylureas, exhibit herbicidal activity by inhibiting this enzyme[7–10]. In the case of sulfonylureas, inhibition of bacterial AHAS appears to be active site directed, since the herbicide competes for the second pyruvate binding site[11].

In microorganisms, as many as six isozymes of AHAS have been described which have different sensitivity to feedback inhibition by amino acids[4,6,12–17]. Similarly, differences in the sensitivity of AHAS isozymes to sulfonylureas have been found in bacteria[9]. In plants, however, isozymes of AHAS have never been reported.

Speculation on the presence of different isozymes in plants has been based on differential sensitivity of the enzyme to inhibition by the branched chain amino acids at different pH values[2,18]. In this report, we present evidence for different forms of AHAS, based on differences in the chromatographic separation and physical and kinetic characterization of AHAS from Black Mexican Sweet corn cells.

MATERIALS AND METHODS

Black Mexican Sweet corn cells

Embryo-derived cell suspension cultures of *Zea mays* var. Black Mexican Sweet, were obtained from Molecular Genetics (Minnetonka, MN, U.S.A.) and cultured on Murashige and Skoog salts[19,20] with 2% (w/v) sucrose, 500 mg/l thiamine, 2 mg/l (2,4-dichlorophenoxy)acetic acid and 150 mg/l asparagine with shaking at 100 rpm in the dark at 22°C. Cells were harvested on day 7 by filtration through a nylon cloth, washed with deionized water and squeezed to remove excess water. Cells were then used for extraction or frozen in liquid nitrogen and stored at −70°C until used. There was no apparent loss of activity of AHAS during storage at −70°C.

AHAS assay

The AHAS assay described here is a modification of an assay procedure described previously[2]. AHAS activity was measured by estimation of the product, acetolactate, after conversion by acid decarboxylation to acetoin. Standard reaction mixtures contained the enzyme in 50 m*M* potassium phosphate buffer (pH 7.0) containing 100 m*M* sodium pyruvate, 10 m*M* magnesium chloride, 1 m*M* thiamine pyrophosphate (TPP) and 10 μM flavin adenine dinucleotide (FAD). This mixture was incubated at 37°C for 1 h. The reaction was stopped with the addition of sulfuric acid to make a final concentration of 0.85%. The reaction product was allowed to decarboxylate at 60°C for 15 min. The acetoin formed was determined by incubating with creatine (0.17%) and 1-naphthol (1.7%) by the method of Westerfeld[21]. Appropriate checks of direct acetoin formation during the enzyme assay were made.

Protein determination

Protein concentrations were determined by the Bradford method[19] according to the protocol supplied by the manufacturer (Bio Rad, Richmond, CA, U.S.A.).

Determination of molecular weight (mol. wt.)

Enzyme preparation (200 μl) was applied to a Waters Protein Pak 300 SW gel filtration HPLC column (30 cm × 7.5 mm I.D.) which had been pre-equilibrated with 100 m*M* sodium sulfate, 20 m*M* sodium dihydrogenphosphate (pH 7.0) containing 5 m*M* pyruvate, 5 m*M* EDTA and 5 μM FAD. Protein was eluted with the same buffer at a flow-rate of 0.5 ml/min. Fractions (0.5 ml) were collected and analyzed for AHAS activity. The proteins (and their molecular weights) used for calibration were α-amylase (mol.wt. 200 000), gamma globulin (158 000), aldolase (158 000), alcohol dehydrogenase (150 000), bovine serum albumin (67 000), ovalbumin (43 000), myoglobin (17 000) and ribonuclease A (13 700).

Enzyme extraction

For the extraction of AHAS, the cells were powdered in liquid nitrogen and

then homogenized in 100 m*M* potassium phosphate buffer (pH 7.5) containing 10 m*M* pyruvate, 5 m*M* magnesium chloride, 5 m*M* EDTA, 100 μM FAD, 1 m*M* valine, 1 m*M* leucine, 10% (v/v) glycerol and 10 m*M* cysteine. The homogenate was filtered through a nylon cloth (53-μm mesh) and centrifuged at 25 000 *g* for 20 min. The supernatant fraction was brought to 50% saturation with respect to ammonium sulfate and allowed to stand for 20–30 min on ice. It was then centrifuged at 25 000 *g* for 20 min and the supernatant was discarded. The ammonium sulfate pellet was used immediately or frozen with liquid nitrogen and then stored at −20°C until used.

Sephadex G-25 desalting

The ammonium sulfate pellet collected from the previous step was dissolved in appropriate buffer, then loaded onto a bed of Sephadex G-25 (41 cm × 2.5 cm I.D., coarse) pre-equilibrated with the equilibration buffer used for chromatofocusing or chromatography on Mono Q and eluted with the equilibration buffer. The protein fraction was monitored by absorbance at 280 nm and collected for further chromatography.

Chromatofocusing

Chromatofocusing was performed in a polybuffer exchanger 94 column (25 cm × 1 cm I.D.) with a gradient of pH 7 to 4, following the Pharmacia protocol. The column was equilibrated with degassed 20 m*M* imidazole buffer (pH 7.4), containing 5 m*M* pyruvate, 10 μM FAD, 1 m*M* TPP and 1 m*M* magnesium chloride. Desalted enzyme from the previous step was loaded on the column which was then washed with 40 ml of equilibration buffer and eluted with polybuffer 74–hydrochloric acid (pH 4) (1:8) containing 5 m*M* pyruvate, 10 μM FAD, 1 m*M* TPP and 1 m*M* magnesium chloride. Fractions of 5 ml were collected and their pH values determined. The pH of each fraction was then adjusted to pH 7.0 and assayed for AHAS activity. The fractions containing AHAS activity were pooled and concentrated by ultrafiltration, using an Amicon ultrafiltration cell, containing a PM 10 membrane at a pressure of 25 p.s.i.

Anion-exchange chromatography on mono Q

Protein pellets obtained after ammonium sulfate precipitation were dissolved in 25 m*M* phosphate buffer (pH 7.0), containing 5 m*M* pyruvate, 5 m*M* EDTA and 5 μM FAD, and desalted on a PD 10 Sephadex G-25 column, as described previously. The desalted protein was loaded onto a Pharmacia Mono Q HR 5/5 column (5 × 0.5 cm) pre-equilibrated with the above buffer [flow-rate: 1 ml/min; Pharmacia's fast protein liquid chromatography (FPLC) system]. The column was washed with 5 ml of equilibration buffer and eluted at 1 ml/min with a linear 20-min gradient of 0–0.5 *M* potassium chloride in equilibration buffer. Fractions of 1 ml were collected and assayed for AHAS activity. Fractions containing the enzyme activity were pooled and concentrated by ultrafiltration as described above.

RESULTS

Separation of two forms of acetohydroxyacid synthase

Chromatofocusing of partially purified AHAS yielded two peaks of activity

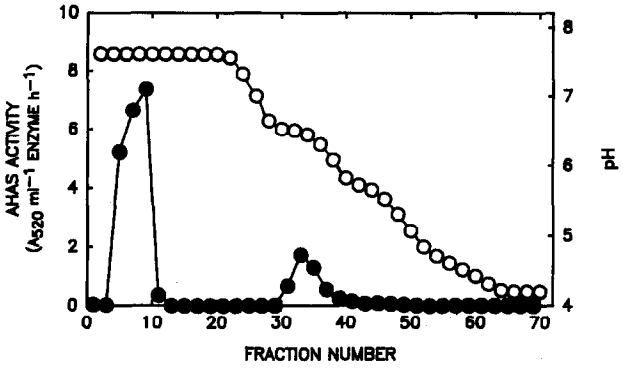

Fig. 1. Chromatofocusing of acetohydroxy acid synthase on a Polybuffer Exchanger 94 column. Details of the chromatographic conditions are described under Materials and methods. ● = AHAS activity; ○ = pH.

(Fig. 1). The major peak of activity (designated AHAS I) did not bind to the polybuffer exchanger 94. This fraction contained about 90% of the total AHAS activity recovered. During a pH gradient elution, a minor peak of AHAS activity (designated AHAS II) was found which contained nearly 10% of the total AHAS activity recovered. The properties of the two peaks of AHAS activity were significantly different. AHAS I was sensitive to inhibition by leucine + valine as well as by imazapyr. On the other hand, AHAS II was completely insensitive to leucine + valine but was even more sensitive to inhibition by imazapyr than AHAS I. Further characterization revealed differences in their molecular weights as well as in other kinetic properties which will be discussed later. The overall recovery of enzyme in this step was 50–60%. This pattern of chromatography was not altered by the scale of chromatography (1–100 mg protein) or by altering the protein to resin bed volume ratio (0.1–1 mg protein per ml resin).

Two similar peaks of AHAS activity with similar properties as described above were also seen after anion-exchange chromatography on Mono Q (Fig. 2). However,

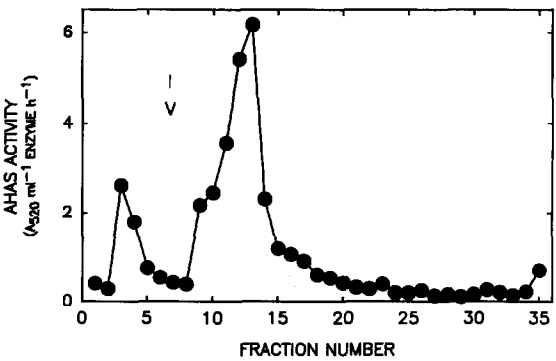

Fig. 2. Chromatography of acetohydroxy acid synthase on a Mono Q HR 5/5 column. Arrow indicates the beginning of salt gradient elution. Details of the chromatographic conditions are described under Materials and methods.

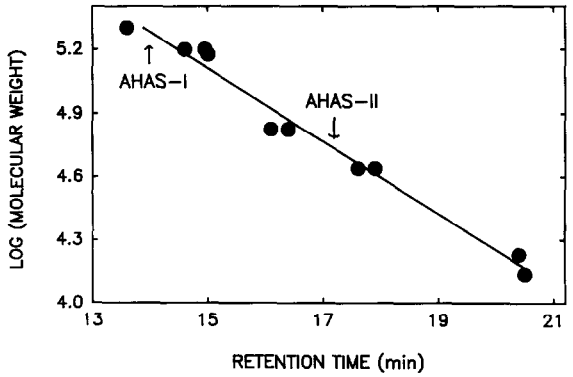

Fig. 3. Estimation of native molecular weight of AHAS I and AHAS II by gel filtration on a Waters Protein Pak SW 300 column. Details of the chromatographic conditions are described under Materials and methods. The standard curve shown here was obtained by running various standards either singly or in a mixture. The molecular weights of AHAS I and AHAS II shown here represent the average of at least three separate runs.

the elution pattern of the two peaks was reversed. The minor peak, AHAS II, was found in the unbound protein fractions whereas the major peak, AHAS I, was eluted during gradient elution. The proportion and recovery of enzyme activity of the two peaks of AHAS activity were similar to those observed during chromatofocusing. Rechromatography of these two peaks separately on the Mono Q column gave only one peak of AHAS activity each at their original retention times.

Molecular weight

The native molecular weights of AHAS I and AHAS II (separated by chromatofocusing or amino-exchange chromatography on Mono Q) were estimated to be 193 000 and 55 000, respectively, by chromatography on a high-performance liquid chromatography (HPLC) gel filtration column (Fig. 3).

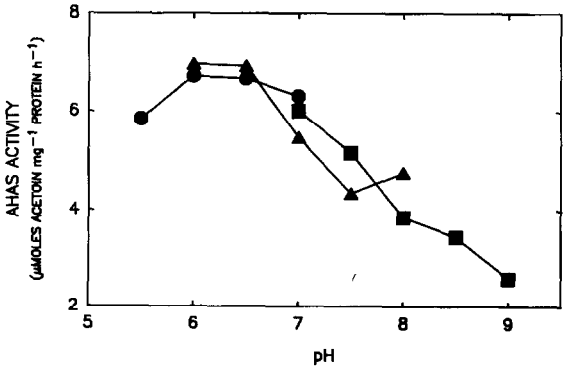

Fig. 4. pH optimum of AHAS I. AHAS was assayed under the standard reaction conditions as described under Materials and methods, except that the pH of the assay was varied using the buffers indicated. ● = MES; ▲ = phosphate; ■ = Tris.

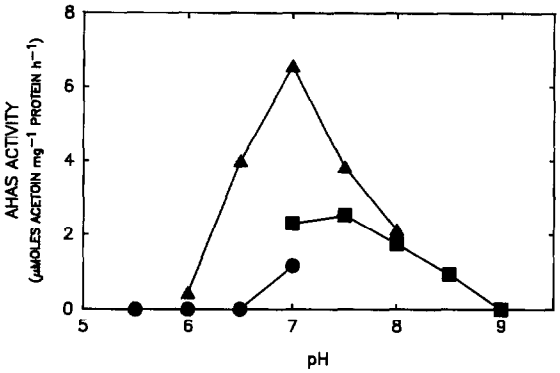

Fig. 5. pH optimum of AHAS II. AHAS was assayed under the standard reaction conditions as described under Materials and methods except that the pH of the assay was varied using the buffers indicated. ● = MES; ▲ = phosphate; ■ = Tris.

pH optima

Both forms of AHAS after chromatofocusing were assayed over a range of pH values using three different buffer systems. As shown in Fig. 4, there is a broad pH optimum for AHAS I between pH 6–7. The type of buffer system used did not affect the results. In contrast, AHAS II had a very distinct pH optimum at pH 7.0 with phosphate buffer (Fig. 5) but showed very little activity in the presence of morpholinoethanesulphonic acid (MES) or Tris.

Pyruvate saturation

The pyruvate saturation curves of AHAS I and AHAS II are hyperbolic (Figs. 6 and 7). Both forms of AHAS appear to be saturated at about 100 m*M* pyruvate. The Hanes–Woolf plot of the data gave a K_m value of 5 m*M* pyruvate for AHAS I and 8 mM for AHAS II (Figs. 6 and 7; Table I).

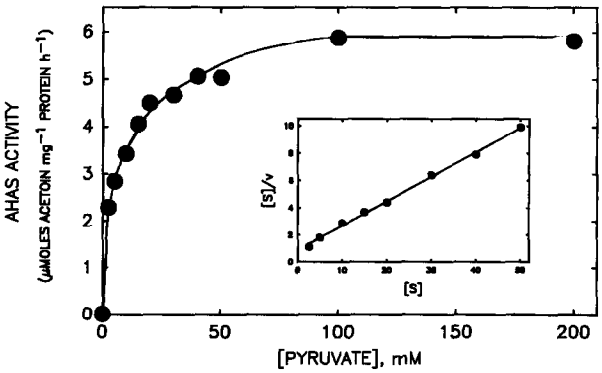

Fig. 6. Substrate saturation curve for pyruvate with AHAS I. AHAS was assayed under the standard reaction conditions as described under Materials and methods except that the concentration of pyruvate was varied as indicated. The insert is an [*S*]/*v versus* [*S*] plot of the data.

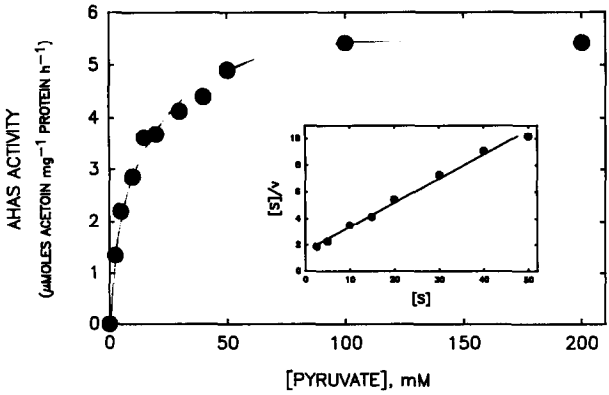

Fig. 7. Substrate saturation curve for pyruvate with AHAS II. AHAS was assayed under the standard reaction conditions as described under Materials and methods except that the concentration of pyruvate was varied as indicated. The insert is an $[S]/v$ *versus* $[S]$ plot of the data.

Inhibition by valine, leucine and isoleucine

The two forms of AHAS were assayed in the presence of valine, leucine and isoleucine, singly or in combination. The degree of inhibition was compared at a concentration of 1 m*M* for each amino acid. The results presented in Table II show that there is little or no inhibition of AHAS II by these amino acids. On the other hand, AHAS I was inhibited by each of these amino acids, either singly or in combination. A combination of leucine and valine gave the highest overall inhibition (66%) of AHAS I. The inhibition of these two forms of AHAS was also examined at varying concentrations of leucine and valine (Fig. 8). Once again, leucine and valine did not inhibit the activity of AHAS II. In contrast, AHAS I activity was significantly inhibited by leucine and valine with 50% inhibition ($I_{0.5}$) at about 0.1 m*M* of each amino acid (Fig. 8).

TABLE I

SUMMARY OF THE PHYSICAL AND KINETIC PROPERTIES OF AHAS I AND AHAS II

In order to obtain the inhibition constants ($I_{0.5}$) for different inhibitors, AHAS I and AHAS II were assayed under standard reaction conditions as described under Materials and methods in the presence of varying concentrations of different inhibitors. In the case of leucine + valine, the values represent concentrations for each amino acid.

Property	*AHAS I*	*AHAS II*
Molecular weight	193 000 ± 21 000	55 000 ± 16 000
pH optima	6–7	7
K_m for pyruvate (m*M*)	5	8
$I_{0.5}$		
(i) leucine (m*M*) + valine (m*M*)	0.1	*
(ii) imazapyr (μ*M*)	2.0	1.5
(iii) sulfometuron methyl (n*M*)	10.0	10.0

* Less than 10% inhibition.

TABLE II

THE EFFECTS OF VALINE, LEUCINE, AND ISOLEUCINE ON AHAS ACTIVITY

AHAS was assayed under the standard reaction conditions as described under Materials and methods.

Form of AHAS	*Percent inhibition caused by various amino acids, each at a concentration of 1 mM*						
	Leu	*Val*	*Ile*	*Leu Ile*	*Val Ile*	*Leu Val*	*Leu Val Ile*
AHAS I	34	34	16	59	50	66	63
AHAS II	4	0	0	0	10	8	0

Inhibition by herbicides

Inhibition of the two forms of AHAS by two different classes of herbicides was also examined. The herbicides used included an imidazolinone (imazapyr; Fig. 9) and a sulfonylurea [sulfometuron methyl (SM); Fig. 10]. Of the two, SM was a more potent inhibitor of both AHAS I and AHAS II. There were significant differences in the kinetics of inhibition of AHAS I and AHAS II. AHAS II was much more sensitive to these herbicides than AHAS I. These herbicides caused a complete or near complete inhibition (>90%) of AHAS II. On the other hand, the maximum inhibition of AHAS I caused by imazapyr or SM was approximately 70%. In spite of these differences, the inhibition constants for imazapyr or SM were the same for both isozymes (Figs. 9 and 10; Table I).

DISCUSSION

This report presents the first biochemical evidence for the existence of two forms of acetohydroxyacid synthase in a plant source. The two forms were well

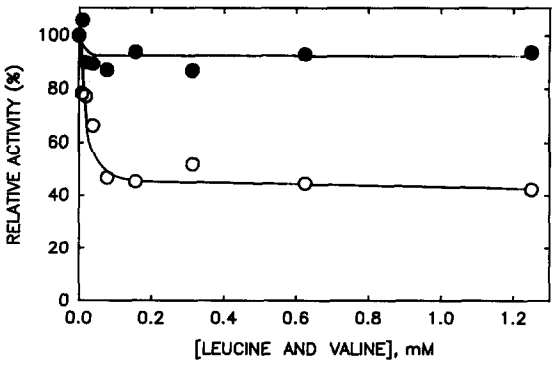

Fig. 8. Inhibition of AHAS I (○) and AHAS II (●) by leucine and valine. AHAS activity was assayed under the standard reaction conditions as described under Materials and methods in the presence of varying concentrations of leucine + valine as indicated. The concentrations indicated here represent the concentrations of each amino acid.

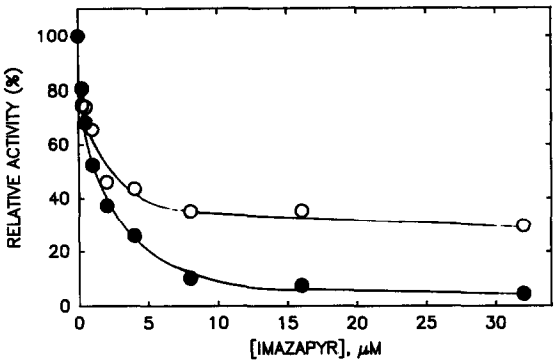

Fig. 9. Inhibition of AHAS I (○) and AHAS II (●) by imazapyr. AHAS activity was assayed under the standard reaction conditions as described under Materials and methods in the presence of varying concentrations of imazapyr as indicated.

separated by chromatofocusing as well as by anion-exchange chromatography on Mono Q. Partial separation of the two forms was also observed during chromatography on hydroxylapatite as well as during hydrophobic interaction chromatography (data not shown). Rechromatography of the two separated forms back on the Mono Q column gave a single peak of AHAS activity at their original elution volumes. This suggests that the two peaks are not artifacts of chromatography. The two forms of AHAS differed significantly in their molecular weight (Fig. 3), inhibition by amino acids (Fig. 8; Table I and II), inhibition by herbicides (Figs. 9 and 10; Table I), pH optima and their preference for buffer (Figs. 4 and 5; Tables I and II).

At this stage, our data are insufficient to determine the origin of the two enzyme forms. The two forms of AHAS may be two separate isozymes. However, a number of other possibilities exist. It is possible that the two types of AHAS are the monomeric and multimeric forms of the same enzyme. Another explanation is that the forms represent the free enzyme form as well as associated with other proteins that regulate its activity *in vivo*. To resolve this question, detailed analysis of the amino

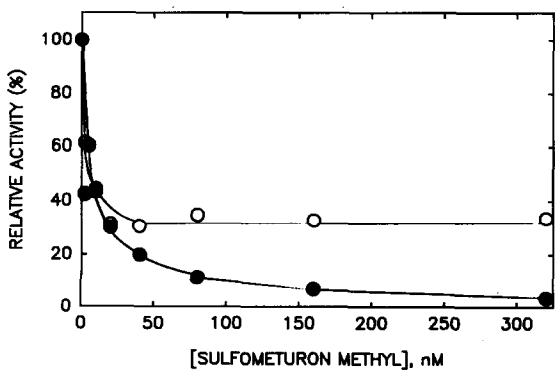

Fig. 10. Inhibition of AHAS I (○) and AHAS II (●) by sulfometuron methyl. AHAS activity was assayed under the standard reaction conditions as described under Materials and methods in the presence of varying concentrations of sulfometuron methyl as indicated.

acid sequences of these polypeptides, or of the nucleotide sequences coding for them, will be required.

The chromatographic behavior of the proteins on chromatofocusing and Mono Q is of interest. Based on the chromatofocusing results, AHAS I appeared to have a p*I* higher than 7.4 (Fig. 1) and, therefore, it should not have bound to Mono Q resin at pH 7.0. However, this proved to be incorrect (Fig. 2). Similarly, AHAS II appears to have a p*I* of 6.0 (Fig. 1) but did not bind to Mono Q at pH 7.0 (Fig. 2). This shows that binding of proteins to ion exchange columns may involve additional types of interactions other than ionic. Similar exceptions have been documented in the literature[22,23].

Many microorganisms regulate carbon flow in branched biosynthetic pathways by having multiple isoenzymic forms of a regulatory enzyme. Typically, each isoenzyme is feedback regulated by the end product of the different pathways, diverging from a metabolic branch point. This prevents complete inhibition of the pathway flux by one end product, the consequence of which could be starvation for the other end products. Complete inhibition is achieved only through the additive effect of more than one inhibitory end product[24–27]. The two forms of AHAS in the present case does not fit this model, because one enzyme (AHAS I) is regulated by the end products of both pathways (valine, leucine and isoleucine) and the other enzyme (AHAS II) is apparently unregulated. Similar observations have been made in the aromatic amino acid biosynthetic pathway of plants[28–30]. Experiments are in progress to elucidate the nature and role of these forms of AHAS.

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