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FOUR LYSOZYMES FROM LATEX OF *ASCLEPIAS SYRIACA*

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Key Word Index—*Asclepias syriaca*; Asclepiadaceae; lysozyme; latex.

Abstract—Four lysozymes have been purified to homogeneity from the latex of *Asclepias syriaca* (milkweed). They have M_rs of about 28 000. The amino acid compositions of the four enzymes are different, but two are apparently related. The lysozymes have different sensitivities to the inhibitor histamine; all are relatively insensitive to *N*-acetyl-D-glucosamine and two have chitinase activity.

INTRODUCTION

Asclepias syriaca L. is a latex-bearing plant growing as a weed (milkweed) throughout much of North America. During the isolation of two families of cysteinyl proteases from that latex [1–3] significant lysozyme activity to cell walls of *Micrococcus lysodeikticus* was observed. As this is relevant to an examination of the biochemical roles of latices which is of interest in this laboratory [4], and comparatively few lysozymes have been isolated as homogeneous proteins from plants, I have purified the lysozymes of *A. syriaca*. Four such enzymes were obtained in homogeneous states and characterized.

Lysozymes have been purified from latices of *Papaya carica* [5], *Hevea brasiliensis* [6] and an unidentified *Ficus* [7]. Lysozymic activities have also been reported in other plants and plant cells [8]. The enzymes of that group which were characterized, have M_rs of 25–30 000 and display compositional similarities [5–8]. They are also distinct from the avian and mammalian enzymes in showing higher rates of chitinase activity [5, 7] and in apparently not carrying essential tryptophan residues in their active sites [7, 9].

RESULTS AND DISCUSSION

The purification of the four lysozymes from latex of *A. syriaca* is described in the Experimental.

While the Bio-Sil TSK 250 is a size-exclusion column, the HPLC separation described here (Fig. 1) cannot be based on M_r: from calibration with known proteins (ribonuclease, carbonic anhydrase, ovalbumin and bovine serum albumin) the R_ts listed would correspond to M_rs of 60, 32, 16 and 6000 respectively. No such heterogeneity was evident on gel-filtration using Bio-Gel P100 in acetate buffer, in the separation step preceding the HPLC. The lysozyme weight was then estimated as 28 000 [10] with slight assymetry in the elution profile as noted. Following the HPLC treatment described, recycling in the same system yielded single symmetrical peaks for each of the R_ts listed.

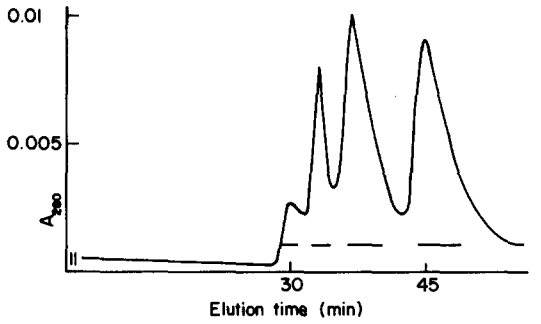

Fig. 1. HPLC elution profiles of lysozymes, from *A. syriaca* latex, on Biosil TSK-250, in 200 mM potassium phosphate, pH 7.0, at 21°. Pools were made as indicated by the horizontal lines.

SDS gel electrophoresis (Fig. 2) shows that all four lysozymes have weights of 30 000 which is in agreement with results reported above from gel-filtration on Bio-Gel P100. Comparable weights have been reported for other lysozymes from plants [6–9]. Isoelectric focussing shows (Fig. 3) that all four lysozymes have major components of pI greater than 9.3. Minor bands are also observed: at 9.3 (lysozymes $_{1,3}$ and $_4$) and 9.1 (lysozymes $_{1,3}$).

The milkweed enzymes have maximum lysozyme activities at pH 4.6 in conventional 'bell' shaped curves, and attain maximum activity at an ionic strength of 100 mM. The chitinase activities of these enzymes are maximal between pH's 5.5 and 8.5 (compare 7, 8).

The amino acid compositions of the lysozymes are presented in Table 1. In none of the four enzymes could tryptophan residues be detected by the three techniques used [11–13]. They resemble, then, other lysozymes from plants in not being dependent on the presence of that residue for activity, in contrast with, for example, avian lysozymes [2, 9]. Further evidence for this was observed when a four-fold molar excess of *N*-bromosuccinimide was without effect on the enzymatic activities of lysozymes$_{1-4}$. No glucosamine or galactosamine was

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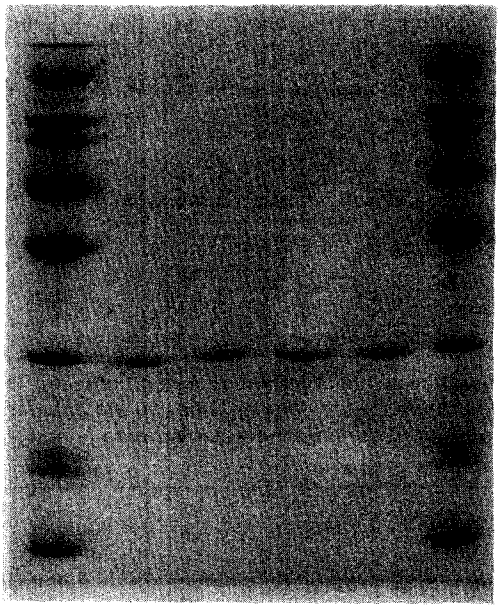

Fig. 2. SDS gel-electrophoresis on the 'PhastGel' system: channels contained (left to right): lysozymes$_{1-4}$ flanked by reference samples containing egg white lysozyme (M_r 14400) soybean trypsin inhibitor (21500); bovine carbonic anhydrase (31000); egg white ovalbumen (66200); phosphorylase a (97400); β-galactosidase (116000) and myosin (200000); reading bottom to top.

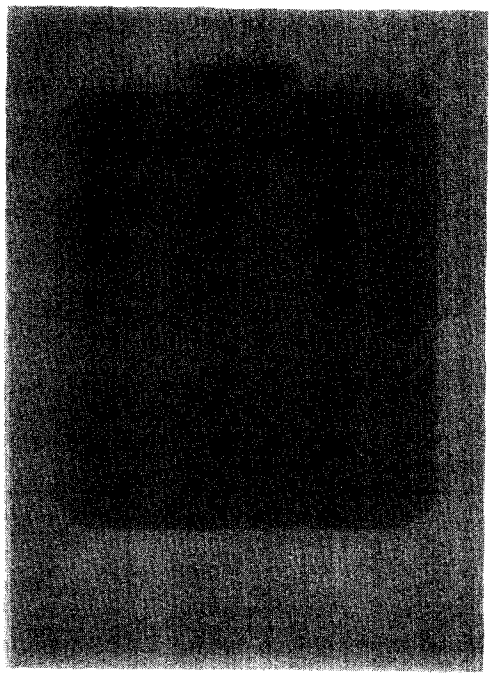

Fig. 3. IEF gel-electrophoresis on the 'PhastGel' system: lysozymes$_{3,4}$ and $_{1,2}$ (left to right) are shown between reference mixtures composed of (from bottom) amyloglucosidase (pI 3.5); soybean trypsin inhibitor (4.55); β-lactoglobulin (5.2); bovine carbonic anhydrase (5.85); horse carbonic anhydrase (6.55); horse myoglobins (6.85; 7.35); lentil lectins (8.15; 8.45; 8.65) and trypsinogen (9.3).

Table 1. Amino acid compositions of four lysozymes from latex of *A. syriaca*

Residue	Residue/mol (% wt)				
Lysozyme:	1	2	3	4	e.w. [19]*
Cys	13 (4.8)	10 (3.6)	13 (4.9)	14 (5.1)	6
Asp	33 (13.4)	25 (10.3)	31 (12.1)	32 (13.1)	16
Thr	16 (5.6)	7 (2.4)	17 (5.9)	17 (6.2)	5
Ser	15 (4.8)	18 (5.5)	19 (5.6)	15 (4.8)	8
Glu	19 (8.6)	34 (15.6)	19 (8.2)	27 (12.7)	4
Pro	15 (5.2)	7 (2.6)	20 (6.6)	18 (6.3)	2
Gly	21 (4.3)	36 (7.3)	31 (6.0)	30 (6.2)	9
Ala	11 (2.8)	16 (4.1)	20 (4.7)	18 (4.5)	9
Val	13 (4.5)	20 (7.1)	12 (4.1)	10 (3.6)	5
Met	1 (0.43)	2 (0.91)	2 (0.91)	1 (0.55)	2
Ile	15 (6.1)	12 (4.9)	11 (4.4)	9 (3.6)	5
Leu	11 (4.5)	10 (4.1)	12 (4.7)	11 (4.4)	6
Tyr	12 (7.0)	17 (10.0)	15 (8.3)	13 (7.3)	2
Phe	21 (11.1)	9 (4.6)	14 (7.0)	11 (5.9)	2
His	1 (0.68)	2 (1.0)	4 (1.8)	5 (2.2)	1
Lys	22 (10.0)	16 (7.3)	11 (4.6)	11 (4.9)	5
Arg	7 (3.9)	13 (7.0)	15 (8.2)	12 (6.6)	9
Trp	0	0	0	0	5

*Egg white lysozyme (chicken).

found in the lysozymes, which are, then, probably not glycoproteins.

Analysis [14] of the data of Table 1 confirmed that lysozymes $_{3,4}$ are related, though these milkweed enzymes differ in composition from other plant lysozymes [6–9].

Histamine is an inhibitor of both avian and plant lysozymes [7] and its effects on the enzymes from *Ascle-*

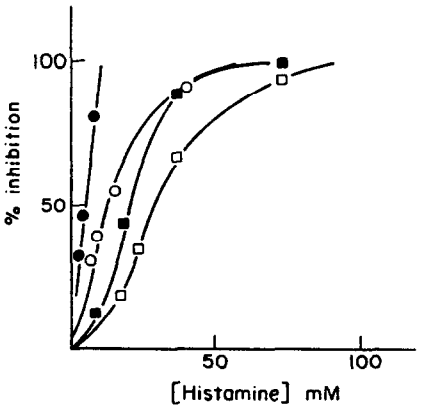

Fig. 4. Per cent inhibition of *A. syriaca* lysozymes, by histamine, in 100 mM sodium acetate buffer, pH 4.6 at 21°. Lysozyme$_{-1}$ —●, $_{-2}$—○, $_{-3}$—■, $_{-4}$—□.

pias syriaca latex are shown in Fig. 4. It is evident that the four lysozymes described here are significantly different from each other in their reactivities with histamine.

N-Acetyl-D-glucosamine is a powerful inhibitor of avian lysozymes, but less effective with those from plants [7]. The results obtained in this work agree with that observation: at 400 mM, *N*-acetyl-D-glucosamine caused only 13% inhibition of lysozyme$_1$, 10% inhibition of lysozyme$_2$, and respectively 13 and 18% inhibitions of lysozymes$_{3,4}$ (cf. [7]).

Lysozymes may also catalyse hydrolysis of chitin, and both the papaya and fig enzymes have greater chitinase activity than the egg white enzyme [7]. Comparable results for the *A.* lysozymes are summarized in Table 2. Only two are chitinases and only lysozyme$_4$ is more active than egg white lysozyme in this property.

EXPERIMENTAL

Reagents. Except where noted, all reagents were of analytical grade. Sigma Chemical Co., St. Louis, MO supplied the dried cells of *Micrococcus lysodeikticus*, histamine and *N*-acetyl-D-glucosamine. The reagents for preparing, running and staining the homogeneous gels, as well as the Bio-Sil TSK-250 HPLC column and guard column, with the Bio-Gel P100 were from Bio-Rad Laboratories, Richmond, CA. Pharmacia (Canada) Ltd., Montreal supplied the reagents for the 'PhastGel' electrophoresis system, and the Sepharose 2B. The CM-52 cellulose was from Whatman Ltd., Maidstone, England; the methane sulphonic acid was provided by Pierce Chemical Co., Rockford, IL, and the Amicon Corporation, Danvers, MA supplied the Diaflo membranes. Latex from *Asclepias syriaca*, which was growing wild in fields in west Quebec, was collected from incisions in the plant stems. The milky liquid was then centrifuged at 20 000 *g* for 1 hr at 4° and the clear aq. intermediate layer ('serum') stored, frozen at −10°, until required.

Lysozyme activity. The procedure used was that of ref. [9]: 10 mg dried cells of *M. lysodeikticus* were suspended in 100 ml 100 mM sodium acetate, pH 4.6, and initial rates of digestion at 37° were measured turbidimetrically, the results being expressed as units of A_{440}/min/ml enzyme soln. Digestion times and enzyme vols were adjusted to ensure measurements of initial rates.

Chitinase activity. Colloidal chitin was prepared and used in digestions at 37° [7] in 100 mM NaOAc buffer, pH 5.6. This was followed by quantitative analysis for reducing groups by reaction with ferricyanide, and activity was expressed in units of μg glucose equivalents/min/ml enzyme soln [15]. Protein determination was commonly made as A_{280}. Alternatively, the Folin procedure [16] was used with bovine serum albumin as standard.

Gel electrophoresis. Homogeneous gels were run conventionally [17]. For SDS and IEF gels, a Pharmacia Ltd. 'PhastGel' system was used with protocols from that company.

M_r determination. A column of Bio-Gel P100 (1.5 × 100 cm) equilibrated with 100 mM NaOAc buffer, pH 4.6, at 4° was calibrated with bovine plasma albumin, ovalbumin, trypsinogen and egg white lysozyme following Andrews [10].

Amino acid analyses. Hydrolysates were prepared in 6 M HCl under vacuum at 110° for 22 hr. Tryptophan was determined after hydrolysis in 4 M methane sulphonic acid under the same conditions [11], and cysteine as described [12]. Tryptophan was also determined spectrophotometrically after oxidation [13] and after treatment with alkali [18]. Amino acid analysis was on a Durrum D-500 automatic amino acid analyser, which was also used to determine amino sugars after hydrolysis in 4 M HCl for six hr at 110° under vacuum.

Enzyme purification. Latex serum (10 ml) was applied to a column of Sepharose 2B (2.5 × 25 cm) equilibrated with 10 mM Tris–HCl buffer, pH 7.0, at 21°. The cloudy, lysozymically active peak was eluted with some separation from lipid materials, and there was a three-fold increase in lysozyme activity: 1.4 A_{440}/min applied and 4.5 A_{440}/min recovered. Apparently inhibitory material was separated at this stage.

The pooled fractions were absorbed on a column of Whatman CM-52 ion exchanger (3 × 20 cm) which was equilibrated with Tris–HCl, 10 mM, pH 7.0, at 21°. After washing with 100 ml of that buffer, a linear gradient of 200 ml each from 10 mM to 500 mM Tris–HCl, pH 7.0, was applied. Two overlapping peaks of lysozyme activity were separated, and there was recovery of

Table 2. Lysozyme and chitinase activities for four lysozymes from latex of *A. syriaca*

Lysozyme	Lysozyme activity (A_{440}/min/mg)	Chitinase activity (μg glucose equiv./min/mg)
1	0.03	0
2	0.04	0
3	0.04	1.0
4	0.20	4.9
Egg white	84	1.0

7.7 A_{440}/min of the 9.0 A_{440}/min applied (85%). The fractions comprising the two peaks of activity was separately concentrated on a Diaflo PM-10 membrane with complete recoveries of activity.

Those concentrates were then dialysed against 100 mM sodium acetate, pH 4.6, and gel-filtered on Bio-Gel P100 (1.5 × 100 cm) equilibrated with that buffer, at 4°, when shoulders on a single peak of lysozyme from each of the concentrates suggested the presence of several active components. The yields obtained were typically 70% of the applied activity: of 2.7 A_{440}/min applied, 1.9 were recovered. The specific activity, which was not measured before this because of slight cloudiness in the enzyme solutions, was increased seven-fold in the gel-filtration.

The pools from the P100 column were again concentrated on the Diaflo PM-10 membrane, in quantitative yields, and subjected to isocratic HPLC. A Bio-Sil TSK-250 column protected by a guard column was used, after equilibration with 200 mM K-Pi, pH 7.0, at 21°. Separation into four lysozymically active components was observed for each P100 pool, as shown in Fig. 1, with extensive loss of activity. Total recoveries were *ca* 5% of the applied activity, and there was concomitant loss of specific activity; from, for example 2.6 to 0.11. The four peaks (Fig. 1) at R_ts of 30, 33, 37 and 45 min (referred to as lysozymes$_{1-4}$) were assayed as respectively, 0.003, 0.008, 0.01 and 0.01 A_{440}/min/ml. Specific activities, from combined pools, are listed in Table 2. Recycling of the separated lysozymes, on the HPLC, was used as needed to produce enzymes which were homogeneous in that technique, on disc gel electrophoresis, and on SDS gel-electrophoresis (Fig. 2).

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