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Extractant Influence on the Relationship between Extractable Proteins and Cold Tolerance of Alfalfa[1, 2]

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ABSTRACT

The influence of ionic composition and pH of extractant on the relationship between the extracted proteins and the cold tolerance of Vernal and Arizona Common alfalfa (*Medicago sativa* L.) was examined. Five environments were used to induce different tolerance levels. The quantity of protein extracted from plants was influenced by the hardening environment, cultivar, and ionic composition and pH of 29 extractants. Extractants with a pH below 6 generally extracted less protein.

The measured cold tolerance of the plants was correlated with the quantity of protein detected in many of the 14 regions of the electrophoresis gel columns regardless of extractant but was most closely associated with the protein in either region 7 or 8 with nine of ten extractants.

The magnitudes of cultivar and hardening effects on quantities of protein detected on various column regions were influenced by choice of extractant. This suggests that proper extractant selection may be vital for study of isozymes by electrophoresis.

Plant survival in areas where freezing occurs often depends upon the capacity of plants to develop cold tolerance. Cultivars of alfalfa (*Medicago sativa* L.) have different capacities to develop cold tolerance (5). A physiological basis for cold tolerance has been the object of many alfalfa studies and the subject of a recent review (5). Water-extractable proteins have been closely associated with the cold tolerance of alfalfa crown and root (2, 3, 7, 8 12, 13). Differences in pH and ionic concentration of plant sap from cold tolerant and cold-sensitive plants (6, 7) may account for greater extractability of proteins from cold-tolerant plants. Gerloff *et al.* (4), extracted similar amounts of protein with a buffered extractant from cultivars that presumably had different levels of cold tolerance. This could be interpreted to mean that the cultivars contained similar amounts of soluble proteins. A possible alternative interpretation is that choice of buffer ion and pH increased extractability of proteins from the cold-sensitive cultivar more than from the cold-tolerant cultivar.

The purpose of this research was to examine the influence of ionic nature and pH of extractants on the relationship between extractable proteins and cold tolerance. Sources of variation in cold tolerance were cultivars of alfalfa and hardening environments.

MATERIALS AND METHODS

Plant Material. Cans with perforated bottoms and a volume of 3.78 liters were filled with a sandy loam soil and seeded with approximately 50 seeds of cold-tolerant Vernal or cold-sensitive Arizona Common alfalfa (*Medicago sativa* L.). The seedlings were allowed to grow in the greenhouse for 7 months and then were moved outside in April. The plants were allowed to reach a flowering stage before clipping to maintain high food reserve levels. Insects were controlled by spraying with malathion and Sevin.

In late September, the containers were randomly assigned to one of five environments: (*a*) day and night temperatures of 7 and 2 C, respectively, and a photoperiod of 8 hr; (*b*) day and night temperatures of 16 and 10 C, respectively, and a photoperiod of 12 hr; (*c*) natural environment in the field at Morgantown, W. Va.; (*d*) greenhouse conditions maintained for vigorous growth; and (*e*) day and night temperatures of 27 and 21 C, respectively, with a photoperiod of 16 hr. Light in the growth chambers was supplied by six cool-white, 40-w fluorescent lamps and four 60-w incandescent bulbs/chamber. This lighting system provided a light intensity of approximately 12,900 lux at the plant tops. Relative humidity was regulated at approximately 80% in the chambers. A randomized block design was used with three replications for each environmental regime.

After the plants had been subjected to the environments for 44 to 46 days, they were sampled for cold tolerance determinations and protein analyses. Plant roots were trimmed to a length of approximately 2.5 cm and crowns to 5 cm. All dead material and plant leaves were removed from the samples. A 10-g portion of prepared crown and root sample was used to measure cold tolerance using the technique of Dexter *et al.* (2). The remainder of each sample was immediately frozen in liquid N_2, lyophilized, ground in a Wiley mill to pass through a 40-mesh screen, and stored at −20 C in air-tight vials that were enclosed in plastic bags.

Protein Extraction. For protein analysis, 200-mg portions of lyophilized powder were extracted overnight at 0 to 4 C with 10 ml of each of the following extractants: (*a*) glycine-HCl, pH 2, 3, or 4; (*b*) succinate,[6] pH 4, 5, or 6; (*c*) phosphate, pH 6, 7, or 8; (*d*) distilled H_2O, pH 6; (*e*) MES, pH 6.15; (*f*) sucrose, pH 6.3;

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[6] Abbreviations: succinate: succinic acid-NaOH; phosphate: KH_2PO_4-KOH; PIPES: piperazine-N,N'-bis(2-ethane sulfonic acid); borate: boric acid-borax; MOPS: morpholinopropane sulfonic acid; Bicine: N,N-bis(2-hydroxyethyl)glycine; TAPS: tris-(hydroxymethyl)methylaminopropane sulfonic acid; CAPS: cyclohexylaminopropane sulfonic acid.

(*g*) PIPES, pH 6.8; (*h*) tris-HCl, pH 7, 8, or 9; (*i*) borate, pH 7, 8, or 9; (*j*) MOPS, pH 7.2; (*k*) TES, pH 7.5; (*l*) HEPES, pH 7.55; (*m*) Tricine, pH 8.15; (*n*) Bicine, pH 8.35; (*o*) TAPS, pH 8.4;(*p*) glycine- NaOH, pH 9, 10, or 11; (*q*) CAPS, pH 10.4. Concentration of the buffer solutions was 0.05 M and pH was adjusted at 0 to 4 C by adding the indicated acid or base (HCl or NaOH if none indicated) at near final dilution. The solution was then further diluted for exact concentration and the pH was again checked.

Following extraction, the solutions were centrifuged at 12,000*g* for 10 min in a refrigerated centrifuge. The supernatant was decanted and saved for analysis. Protein was precipitated from subsamples of this initial supernatant by addition of 10 ml of 5% trichloroacetic acid to 5 ml of the supernatant. After 30 min, the solution was centrifuged for 10 min at 2,500*g*, and the resulting supernatant was discarded, thus removing the extracting buffers from the system. The precipitated protein was then suspended in sodium hydroxide, diluted and analyzed by the Folin phenol method (10). Similar results were obtained from each of the three experimental replications.

Disc Electrophoresis. Disc electrophoresis was used to detect protein components extracted by the 10 extractants (Table II). Techniques used for polyacrylamide gel column preparation, electrophoresis, staining, destaining, and densitometry of stained columns were described in an earlier report (3).

Densitometric graphs were divided into 14 regions, because low areas or valleys between peaks appeared most consistently at these points (3). Approximate relative mobilities of the major component of each gel column region for distilled H_2O extracts, assuming the origin to be 0 and the center of the fastest moving band to be 10, were as follows: column region 1 = 10.0, 2 = 8.6, 3 = 7.2, 4 = 5.8, 5 = 5.0, 6 = 4.5, 7 = 3.6, 8 = 2.6, 9 = 1.9, 10 = 1.0, 11 = 0.8, 12 = 0.5, 13 = 0.4, and 14 = 0.0. Lines were drawn from these low areas on the densitometric patterns to the integrator blips along the base line with the aid of a T square. Recorded blips under each peak were counted for statistical analysis. Linear regression and simple linear correlation coefficients were used for statistical evaluation of the protein-cold tolerance relationship.

RESULTS

Cold tolerance determinations confirmed our expectations that the cultivars and environments employed would result in a wide and evenly distributed set of cold tolerance values (Fig. 1).

Total Extractable Proteins. The amount of protein extracted from alfalfa was influenced markedly ($P < 0.01$) by the ionic composition and pH of 29 extractants (Table I). From 25 to 50 times more protein was removed with PIPES, pH 6.8 than with glycine-HCl, pH 3 or succinate, pH 4. In the examined pH range of 2 to 11, a major portion of the proteins was less soluble when pH was below 6. Amounts of extracted protein also varied considerably between extractants, even when pH was not a variable. Furthermore, protein removal by the 29 extractants was differentially influenced ($P < 0.01$) by cultivar and environment.

Table I. *Relationship between Plant Freezing Tolerance and Protein Extracted from Crown and Root Samples by Several Extractants*

Regression equation: y = a + bx, where y = % electrolytes exosmosed, x = proteins as mg/g dry tissue.

Extractant	pH	Correlation			x̄
		Coefficient (r)[1]	a	b	
					mg/g
Glycine-HCl	2	0.9169**	72.08	−1.29	18.4
Glycine-HCl	3	0.2643	50.86	−3.17	0.8
Glycine-HCl	4	0.8990**	89.59	−2.13	19.3
Succinate	4	0.4786**	55.01	−2.96	2.2
Succinate	5	0.8345**	96.73	−3.14	15.3
Succinate	6	0.9226**	110.02	−1.98	31.1
Phosphate	6	0.9255**	108.65	−1.83	32.8
H_2O	6	0.9325**	97.11	−1.50	32.4
MES	6.15	0.9209**	109.14	−2.03	29.8
Sucrose	6.3	0.9199**	99.35	−1.50	33.9
PIPES	6.8	0.9258**	175.16	−2.22	57.2
Phosphate	7	0.9435**	109.97	−1.58	38.8
Tris-HCl	7	0.9478**	100.34	−1.69	30.7
Borate	7	0.9515**	98.79	−1.70	29.6
MOPS	7.2	0.9267**	107.71	−1.67	35.5
TES	7.5	0.9408**	106.75	−1.67	34.8
HEPES	7.55	0.9414**	109.13	−1.71	35.5
Phosphate	8	0.9350**	110.92	−1.64	38.0
Tris-HCl	8	0.9461**	106.48	−1.60	36.3
Borate	8	0.9526**	105.38	−1.52	37.4
Tricine	8.15	0.9337**	109.52	−1.62	37.7
Bicine	8.35	0.9300**	107.04	−1.56	37.5
TAPS	8.4	0.9415**	106.18	−1.50	38.5
Tris-HCl	9	0.9144**	106.62	−1.50	38.6
Borate	9	0.9367**	110.75	−1.55	40.3
Glycine-NaOH	9	0.9472**	101.95	−1.53	35.0
Glycine-NaOH	10	0.9375**	113.25	−1.58	41.1
CAPS	10.4	0.9416**	108.31	−1.60	37.5
Glycine-NaOH	11	0.9275**	112.12	−1.57	40.5

[1] Correlation coefficients run with 30 pairs of values including 2 cultivars, 5 environments, and 3 replications.

** Significant at the 0.01% level.

Although protein values ranged from 1 to 57 mg/g tissue, protein concentration was usually proportional to cold tolerance (Table I). Linear correlation coefficients between protein concentration and cold tolerance exceeded 0.9 with all extractants except glycine-HCl, pH 3 or 4, and succinate, pH 4 or 5. These four extractants generally removed smaller amounts of protein than other extractants regardless of cultivar or level of cold tolerance.

The total amount of soluble protein electrophoresed on disc columns was different for the two cultivars (analysis of variance not presented) with all extractants except tris-HCl pH 8, phosphate pH 6 and succinate pH 5 (Table II). Differences between cultivars tended to be greater when protein was extracted with H_2O or extractants at a pH near 7.0, *i.e.* tris-HCl pH 7 and phosphate pH 7. The small amount of protein extracted with succinate pH 5 may account, at least in part, for not detecting a difference in protein level between cultivars. This would not, however, explain the results obtained with tris-HCl pH 8, and

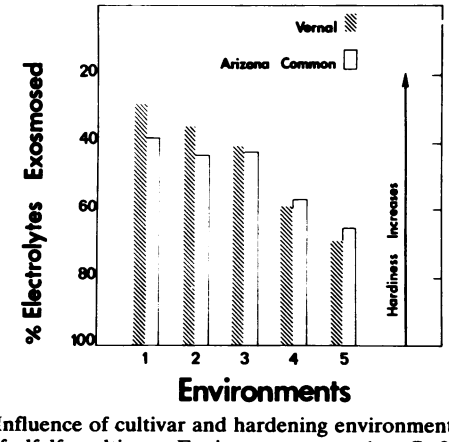

FIG. 1. Influence of cultivar and hardening environment on the cold hardiness of alfalfa cultivars. Environments are: 1 = 7, 2 C; 2 = 16, 10 C; 3 = outside; 4 = greenhouse; 5 = 27, 21 C day, night temperatures.

Table II. *Effect of Extractant, Environment, and Cultivar on Total Protein per Column*

Protein is expressed as integrator counts/gel column. Each column represents 3.5 mg of lyophilized crown and root tissue.

Extractant	pH	Environments									
		7-2 C		16-10 C		Outside		Greenhouse		27-21 C	
		Vernal	Ariz. Common	Vernal	Ariz. Common	Vernal	Ariz. Common	Vernal	Ariz. Common	Vernal	Ariz. Common
Borate	9	409.3	360.0	374.3	309.7	362.3	314.3	220.7	236.3	208.3	225.0
Tricine	8.15	381.3	342.7	352.3	301.3	363.0	308.3	107.3	240.7	210.0	206.7
Tris-HCl	8	365.3	326.7	324.0	278.7	305.0	300.7	191.7	218.3	181.7	185.3
MOPS	7.2	355.7	299.3	311.3	267.3	316.7	276.0	195.7	214.3	180.7	190.7
Phosphate	7	333.0	283.3	320.3	262.3	289.0	263.7	189.3	202.7	171.7	164.7
Tris-HCl	7	350.3	276.7	298.7	214.7	269.3	252.0	167.0	176.7	140.0	155.7
Distilled H_2O		327.7	254.0	263.7	232.3	270.6	241.0	176.3	173.7	142.7	155.3
Succinate	6	287.0	271.3	259.0	205.7	258.0	219.7	173.7	182.3	154.0	142.0
Phosphate	6	281.7	249.7	252.3	220.0	232.7	203.7	156.0	162.3	145.0	154.3
Succinate	5	121.3	137.0	98.3	95.0	94.0	98.3	71.3	77.3	66.0	64.3

our results with this extractant are in agreement with those reported by Gerloff *et al.* (4).

Total amounts of protein on the gel columns were greatly influenced by the five environments to which the plants had been exposed, but the effects were different for the two cultivars. Such interactions were expected and were significant for all extractants except phosphate pH 6 and succinate pH 5 or 6, the extractants that removed less protein.

Electrophoretic Separations. The influence of 10 extractants on protein removed from plant tissues differing in cold tolerance and electrophoresed on 14 column regions is illustrated in Figure 2. Much of the protein was separated into regions 3 through 9 with region 5 generally containing the largest portion. This tendency was more pronounced when investigations were conducted with Arizona Common than with Vernal plants. Protein patterns for Arizona Common plants contained more protein in region 5 and less in regions 3 and 9 than patterns for Vernal plants regardless of extractant. Protein quantities observed on regions 7 and 8 also depended on the cultivar when most extractants were used. Use of any extractant other than succinate pH 5 revealed that the magnitude of cultivar differences in regions 3 and 7 through 9 was influenced by hardening with Vernal plants undergoing much greater increases due to hardening than Arizona Common plants.

Generally small amounts of protein were detected in column regions 1, 2, and 11 through 13. Cultivar and environmental influences detected in these regions were small when most extractants were used.

The quantity of protein in each of the 14 column regions was greater for hardened than for unhardened plants with six of ten extractants. The amounts of protein in regions 2 through 9 increased with hardening regardless of extractant and in regions 10 through 12 with all extractants except succinate pH 5. Although the quantity of protein in column region 4 was strongly influenced by hardening environment, a cultivar difference was detectable only when the extractant was tris-HCl pH 8 or succinate pH 5. In contrast to most observed cultivar differences, Arizona Common exceeded Vernal when the protein in region 4 was compared for these two extractants.

Even when cultivar and hardening effects were consistent for particular column regions, the magnitude of the effects was often markedly different for the ten extractants. Increases in the proteins in column regions 9 through 14 due to hardening were minimized when the extractant was succinate pH 5. Cultivar influence on protein detected in region 8 was reduced when tris-HCl pH 8, Tricine pH 8.1, or succinate pH 5 were utilized in comparison to other extractants. Protein in region 6 did not differ for the two cultivars when extraction was by either of the phosphate buffers, and cultivar influence on the amount of protein in regions 11 and 14 was minimized when tris-HCl pH 8 was used for extracting.

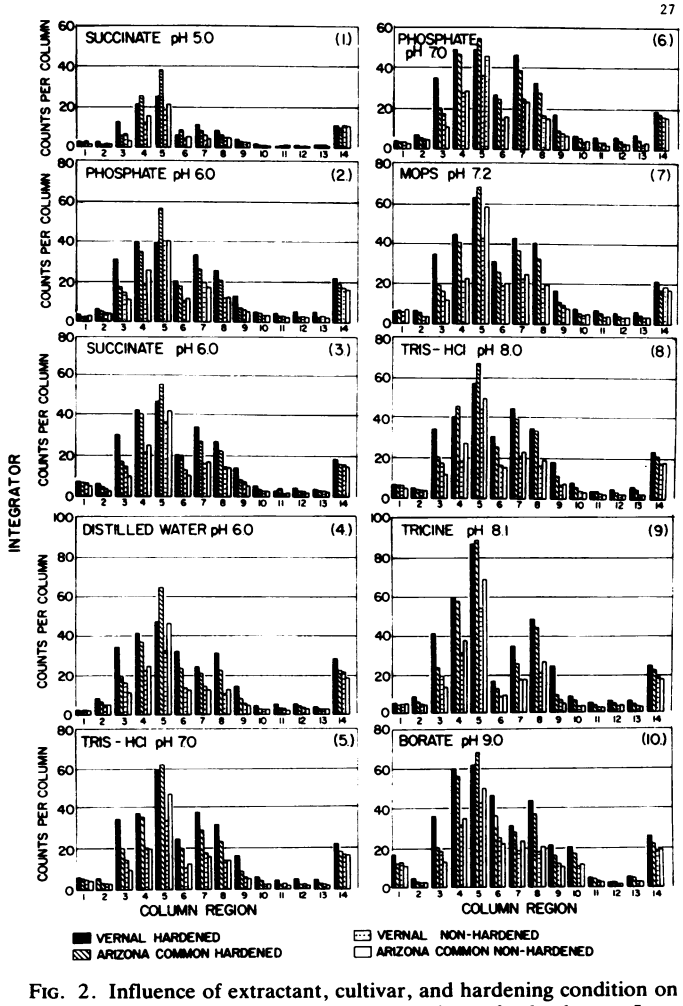

FIG. 2. Influence of extractant, cultivar, and hardening condition on electrophoretic protein separation into 14 regions of gel columns. Integrator counts are proportional to protein quantity.

Association between Cold Tolerance and Different Proteins. Observed differences in cold tolerance were closely related to the quantities of the proteins detected in column regions 2 through 9 regardless of extracting solution and for regions 10 through 12 for all extractants except the pH 5 succinate buffer (Table III). Differences in cold tolerance were most closely

Table III. *Association between % Electrolytes Exosmosed and Protein per Column as Indicated by Correlation Coefficient (r)*

Correlation coefficients were calculated for 30 pairs of observations including 2 cultivars, 5 environments, and 3 replications.

Column region	Extractants									
	Borate pH 9	Tricine pH 8.1	Tris-HCl pH 8	MOPS pH 7.2	Phosphate pH 7	Tris HCl pH 7	Distilled H_2O	Succinate pH 6	Phosphate pH 6	Succinate pH 5
Total	−.92**	−.90**	−.94**	−.91**	−.90**	−.94**	−.94**	−.87**	−.82**	−.72**
1	−.47**	−.03	−.25	−.04	−.23	−.55**	−.18	−.21	−.09	−.22
2	−.66**	−.53**	−.51**	−.57**	−.76**	−.69**	−.75**	−.74**	−.42*	−.54**
3	−.75**	−.73**	−.80**	−.78**	−.76**	−.81**	−.81**	−.77**	−.73**	−.68**
4	−.89**	−.80**	−.77**	−.86**	−.87**	−.88**	−.78**	−.73**	−.73**	−.67**
5	−.64**	−.75**	−.64**	−.57**	−.49**	−.74**	−.53**	−.53**	−.39*	−.45*
6	−.81**	−.70**	−.86**	−.88**	−.85**	−.94**	−.87**	−.66**	−.76**	−.48**
7	−.72**	−.84**	−.90**	−.86**	−.88**	−.92**	−.73**	−.87**	−.83**	−.71**
8	−.93**	−.88**	−.90**	−.88**	−.86**	−.87**	−.90**	−.85**	−.80**	−.73**
9	−.88**	−.81**	−.88**	−.86**	−.75**	−.85**	−.86**	−.77**	−.79**	−.40*
10	−.89**	−.64**	−.82**	−.62**	−.78**	−.80**	−.58**	−.77**	−.61**	−.17
11	−.72**	−.75**	−.49**	−.74**	−.54**	−.59**	−.62**	−.49**	−.43*	−.17
12	−.51**	−.65**	−.73**	−.75**	−.67**	−.69**	−.53**	−.58**	−.57**	−.13
13	−.65**	−.51**	−.83**	−.62**	−.79**	−.75**	−.25	−.49**	−.61**	−.13
14	−.76**	−.84**	−.73**	−.21	−.48**	−.79**	−.58**	−.40*	−.47**	−.10

* Significant at the 0.05 level.

** Significant at the 0.01 level.

associated with protein differences in region 8 when six of the ten extractants were used and with differences in region 7 when three extractants were used. Differences in cold tolerance were also closely associated (r = 0.8 or above) with difference in the proteins in regions 3, 4, 6, 9, and 10 when many of the extractants were used. Correlation coefficients were below 0.6 when cold tolerance was compared to protein differences for column regions 1, 2, 5, 11, or 14 with several of the extractants.

DISCUSSION

This study seemed particularly appropriate because consistent increases in pH of plant sap have been observed during the acquisition of cold tolerance and the rises in pH were greater in cold-tolerant than cold-sensitive cultivars (6, 7, 11, 12). Foliar applications of purines and pyrimidines that increased cold tolerance also tended to increase pH of plant sap (6). The observation in this study that a major portion of the proteins was not soluble below pH 6 is noteworthy because this corresponds to the transitional pH of sap above and below which alfalfa plants generally are cold-tolerant and cold-sensitive, respectively.

Gerloff *et al.* (4) emphasized the importance of using a buffered extractant for soluble protein extraction. It now appears that tris-HCl pH 8 which they used has its own peculiar extraction properties that differ from those of most extractants examined in this study. Lack of cultivar differences in their studies and in ours was not common to all buffered extractants nor was it common to pH of the extracting solution.

The amount and kind of soluble proteins extracted were markedly influenced by ionic composition and pH of the extractants. Such information is pertinent, if not critical, in studies where the importance of proteins is to be evaluated, and especially so where cultivar or environmental relationships are of interest. The selection of an appropriate extractant for such studies could be based upon pH of soluble cytoplasm but this is not known nor can it be readily obtained (J. A. Raven, unpublished data). Moreover, plant sap measurements indicate that differential seasonal shifts may occur in cytoplasmic pH.

Previous investigators (4) using techniques similar to those used in this study have found that some of the protein bands separated by electrophoresis were isozymes. Relative shifts to more stable isozymes may be an integral part of the hardening process. The observed influence of extractants on the magnitude of cultivar differences in the proteins observed on various column regions suggests that extractant selection could markedly alter the results of isozyme comparisons.

These concerns suggest that selection of an extractant be made after some comparisons have been made among extractants to determine their effects on the phenomena under investigation. We have, for example, continued our investigations using three extractants H_2O, tris-HCl pH 7, and borate pH 9) and found that thermal stability of amylase isozymes was differentially affected by the extractants (9). Although the borate extractant removed more amylase, the proteins were not as stable as those removed with H_2O or tris-HCl pH 7.

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