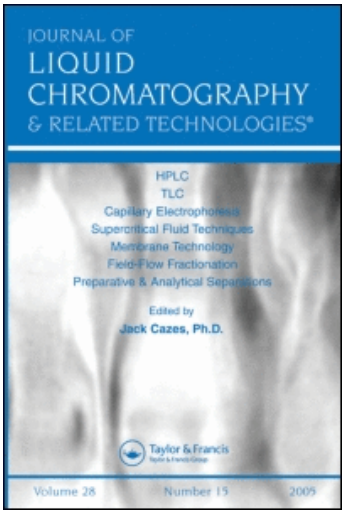

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Egg White Protein Separation Development: Effect of Buffers and Untreated Fused Silica vs. Neutral Covalently Bonded Columns

J. D. McCulloch[a]
[a] Supelco, Incorporated, Beliefonte, Pennsylvania

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EGG WHITE PROTEIN SEPARATION DEVELOPMENT: EFFECT OF BUFFERS AND UNTREATED FUSED SILICA VS. NEUTRAL COVALENTLY BONDED COLUMNS

J. D. McCULLOCH
Supelco, Incorporated
Supelco Park
Bellefonte, Pennsylvania 16823

ABSTRACT

A method for the separation of three egg white proteins, lysozyme, conalbumin, and ovalbumin, was developed based on desired criteria of reproducible baseline separation of all components in under 25 minutes. Fixed parameters were 50cm effective column length, 200 volts/cm field strength. Sodium phosphate, sodium borate, and phosphate/borate combination buffers were compared in different ionic strengths. The performance of bonded phase columns, CElect™-P1 and CElect-H (patent pending) were compared to that of untreated fused silica. The optimal separation was obtained using 100mM phosphate / 60 mM borate buffer, pH 8.0 on the hydrophilic CElect-P1 bonded phase column.

INTRODUCTION

Since its development as a separation technique, one of the greatest difficulties encountered in the separation of proteins by capillary electrophoresis is the adsorption of the proteins, at neutral pH, to the

untreated fused silica surface (1), and the irreproducibility that accompanies this adsorption. Researchers have demonstrated ways to ease this problem by using high pH buffer (above the pI of the protein to reduce ionic attraction) (2), using low pH buffer to protonate the column surface (3), using high buffer concentrations or ionic additives to compete with proteins for silanol sites (2,4,5), and making permanent modifications to the capillary wall (3, 6-12).

Commercially available phase-coated columns reduce the electroosmotic (EEO) flow, while not eliminating the flow completely, by changing the charge density along the wall surface (12). Phases in such columns have been shown to be stable and reproducible under various conditions, including a wide range of pH. Although the number of times a protein will make contact with the wall over the length of the column is small, due to the low diffusivity of proteins (13), those contacts can be of significant duration, depending on phase type and the hydrophobicity of the phase and the proteins involved. The best method for any given analysis may include none, one, or a combination of the previously mentioned options.

Developing a method for capillary electrophoresis (CE) involves more than ensuring an inert surface, it involves the optimization of many variables just as in other separation techniques. In CE, buffer composition, concentration, and pH must be considered, as well as applied voltage, column length, and field strength. Each of these has an independent effect on the separation, while some also exhibit cooperative effects. In untreated fused silica, the effect of changing pH is two-fold, both EEO flow and molecular mobility can change, due to net charge changes. In bonded phase columns, due to the stabilization of EEO flow over a wide pH range, changes in pH are predominently apparent molecular mobility changes (12). This is beneficial when trying to achieve the desired resolution of components with a mixture.

Three major components of egg white protein are lysozyme (pI 11.0, MW 14,000), conalbumin (pI 6.6, MW 77,000), and ovalbumin (pI 4.7, MW

43,500). In free zone electrophoresis, these three compounds would be expected to separate quickly and easily in a common biological buffer at a neutral pH, due to their vast differences in charge and mass. However, in reality, due to electrostatic adsorption of positively charged amino acid residues on a protein to the untreated fused silica wall, the analysis is difficult. The effects of buffer composition, concentration, and pH were investigated for the separation of these proteins. The results were compared for two bonded phases (CElect-P1 and CElect-H), and untreated fused silica.

EXPERIMENTAL

Instrumentation

All CE experiments were performed on an ABI model 270A-HT capillary electrophoresis system, (Applied Biosystems, Foster City, CA). Data collection was performed on an IBM Personal System 2 computer (IBM, Armonk, NY) utilizing Beckman System Gold software and a 406 analog interface module (Beckman Instruments, Fullerton, CA). Bonded phase capillary electrophoresis columns, CElect-P1 and CElect-H (Supelco, Bellefonte, PA), and untreated fused silica (Polymicro Technologies, Phoenix, AZ) of 363 um o.d. and 50 um i.d., were used with total lengths of 85 cm and effective lengths of 50 cm.

Electrophoresis

All protein solutions were injected under vacuum for 1.5 seconds. Protein standards were 1 mg/ml in water; egg whites were diluted 1:20 in water or separation buffer. Several buffer solutions were used to perform analyses: 50 mM and 100 mM sodium borate, pH 8.0; 50 mM and 100 mM sodium phosphate, pH 7.0 (dibasic/monobasic mixtures); 100 mM sodium monophosphate / 100 mM sodium borate, pH 8.0; and 100 mM sodium monophosphate / 60 mM sodium borate, pH 8.0. Applied voltage

was 200 V/cm, detection was at 200 nm, and the separation temperature was 30°C. Bonded phase columns were conditioned per the manufacturer's instructions, and all columns were flushed with 0.1 N sodium hydroxide for 1 minute, followed by 4 minutes of the separation buffer after each analysis.

Reagents

Proteins were purchased from Sigma Chemical Company (St. Louis, MO) as lyophilized powders. Sodium phosphates were also purchased from Sigma. Additional lysozyme was purchased from Calbiochem (San Diego, CA), and sodium borate was purchased from Fisher Scientific (Pittsburgh, PA). All water used was Milli-Q water obtained from a 5-bowl system fed by an RO-6 unit (Millipore Corporation, Milford, MA).

RESULTS AND DISCUSSION

Lysozyme has long been recognized as a difficult protein to analyze, due to its high pI and significant level of hydrophobicity. Because of these characteristics, lysozyme was chosen as the focal point for the separation. Of the commercially available bonded phases, the CElect-P1 and CElect-H columns were chosen based on their characteristics. The CElect-P1 column is a hydrophilic phase column, with only a slight reduction in electroosmotic flow compared to untreated fused silica. The CElect-P1 does, however, demonstrate considerable silanol character. The CElect-H column is a dimethyl (C1) phase, having a slightly hydrophobic nature, but which exhibits the least silanol character available, as evident by the greatly reduced electroosmotic flow compared to untreated fused silica (12).

The CElect-P1 column, due to its hydrophilic phase and fast flow, was investigated initially. Using 50mM sodium phosphate buffer, pH 7.0, lysozyme appeared as only a distortion in the baseline subsequent to the

neutral marker, which was the water used to make the sample. Using a 50mM sodium borate buffer, pH 8.0, a split peak eluted behind the neutral marker. Lysozyme, which is positively charged at both pH 7.0 and 8.0, should elute before the neutral marker. The post- marker elution indicates interaction with the column, causing the zone to be held back. In 100mM sodium phosphate buffer, pH 7.0, lysozyme elutes as a low, broad hump in the baseline, again after the marker. In 100mM sodium borate buffer, pH 8.0, lysozyme eluted as a distinct, but tailing peak, prior to the marker. Raising the concentration of the buffer apparently was reducing the protein's interactions with the column, most probably with siloxane anions, producing better elution.

It has been noted that using buffers with the same concentrations, but which have different anions give different selectivity (14). In an attempt to further improve the results, a combination buffer of 100mM phosphate and 100mM borate was used. However, the joule heat generated by the system at this high strength was beyond the cooling system's dissipating ability, and was evident in unacceptable baseline noise and rise. It is important to note, however, that the lysozyme peak shape was definitely improved over prior analyses. In an attempt to reduce the joule heating, a combination buffer of 100mM phosphate / 60 mM borate, pH 8.0 was used, and yielded not only a distinct lysozyme peak of good shape, but another distinct peak close behind, also possessing good shape. The identity of this peak will be discussed later.

The three component standard sample was then analyzed under the same conditions and yielded separation of all components in less than 20 minutes. The conalbumin, negatively charged under these conditions, elutes with good peak shape as the second of the three proteins, followed by ovalbumin, the most negatively charged protein. The ovalbumin peak appears with a shoulder, an associated impurity, as seen previously (15). Peak identities were confirmed by analyses of individual standard proteins.

To study the effects of high concentration buffers on the overall separation of the three component mix, samples were analyzed in the

100mM phosphate / 100mM borate, pH 8.0 buffer. The heat generated internally was so great the protein peaks degraded to essentially zero response in three analyses. Worth noting however, is that although the peak shapes were very good, the high ionic strength created such an increase in the charge double layer that the electroosmotic flow was slowed to the extent that ovalbumin did not elute within the 25 minute time frame.

The separation of the standard sample on the bare column using the 100mM phosphate / 60 mM borate, pH 8.0 buffer showed severe lysozyme adsorption and slight conalbumin adsorption (Figure 1). The adsorption slowed the lysozyme, resulting in elution after the marker. Subsequent analyses showed the coating effect which occurs with proteins on untreated fused silica. The shape and position of the lysozyme peak improved noticeably with each analysis. Also apparent was the absence of the lysozyme companion peak. The shape of the conalbumin peak improved, predominantly between the first and second runs, while the elution of ovalbumin was relatively unchanged. This was as expected. Although the high ionic strength helped to reduce ionic attraction to the silanols, these conditions did not completely eliminate this interaction. Reproducibility of migration times for each component was compared for three analyses on both the bare and CElect-P1 columns using the 100mM sodium phosphate / 60 mM sodium borate, pH 8.0 buffer. As can be seen in Table 1, the percent relative standard deviation (%RSD) for each component was significantly lower for the CElect-P1 column.

The standard sample was separated under the same conditions on the CElect-H column, and yielded several interesting observations (Figure 2). The EEO flow, already characteristically slow, and further slowed due to the high buffer concentration, yielded only a broad peak for lysozyme in less than 25 minutes. The analysis time was extended to 40 minutes, and although the lysozyme peak shape improved with subsequent analyses, neither of the remaining components migrated past the window. It is apparent that the high ionic strength did not prevent adsorption on this

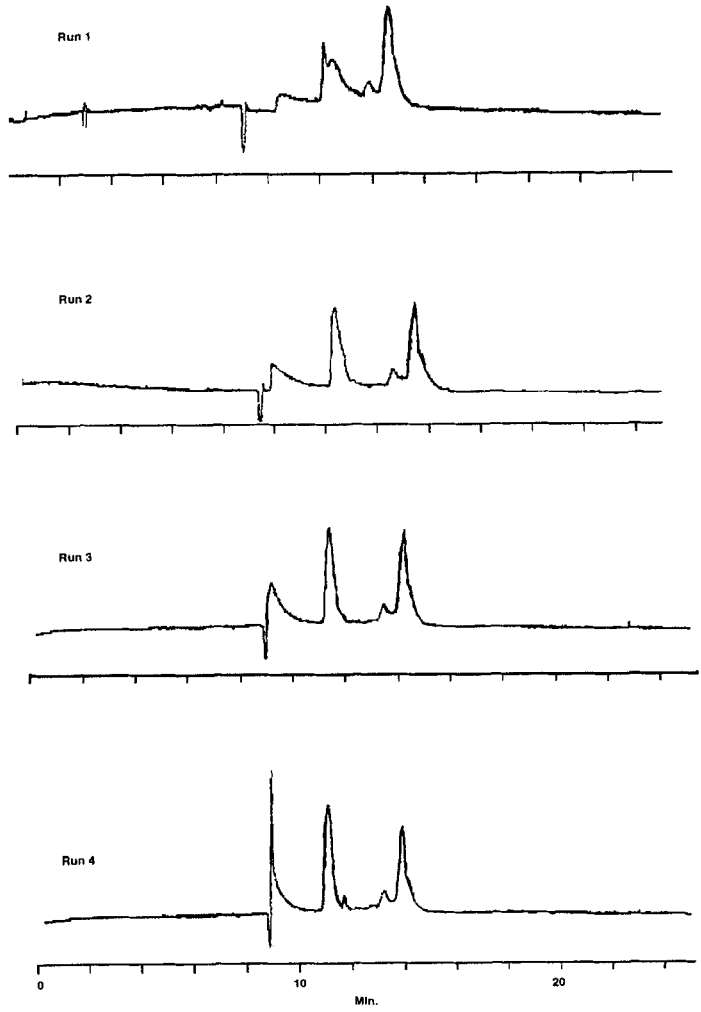

Figure 1: Egg white protein mix on an untreated fused silica column: Analyses 1-4. Buffer: 100mM sodium phosphate / 60 mM sodium borate, pH 8.0. Peaks: 1) lysozyme 2) conalbumin 3)ovalbumin.

TABLE 1

CElect-P1 vs. Untreated Fused Silica Column: Reproducibility of Protein Migration Time

Protein	CElect-P1 Mean*	CElect-P1 %RSD	Untreated Silica Mean*	Untreated Silica %RSD
Lysozyme	9.9	0.47	10.6	1.54
	10.6	0.00	11.2	1.83
Conalbumin	12.9	0.63	14.0	2.76
Ovalbumin	16.8	0.85	18.2	3.76

* in minutes, n=3

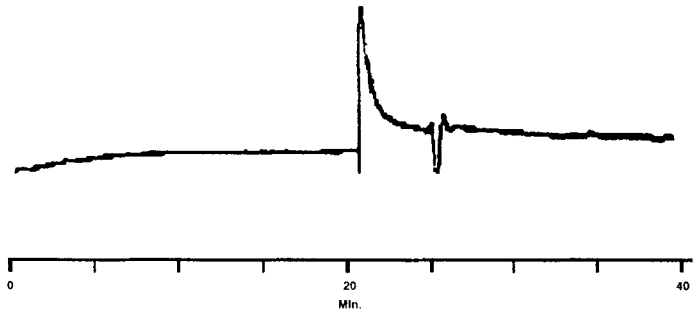

Figure 2: Egg white protein mix on a CElect-H column: 100mM sodium phosphate / 60 mM sodium borate, pH 8.0. Only lysozyme eluted peak in 40 minutes.

column as it had on the CElect-P1 and untreated fused silica columns. This suggests hydrophobic interaction of the protein with the phase as the mode of adsorption, as opposed to ionic interactions. As mentioned previously, although the number of contacts with the wall are low, the contact duration plays a significant role in the elution of lysozyme.

Many efforts were made to determine the identity of the lysozyme companion peak. The lysozyme was analyzed on an isoelectric focusing

gel, to determine sample purity. However, the pI of the lysozyme fell outside the usable range of the gel, and thus no conclusions could be reached. Lysozyme was obtained from another vendor and yielded the same capillary electrophoretic results. Analysis of an egg white sample would ensure pure native lysozyme (17). Chicken egg white was diluted 1:20 in both water and separation buffer. A portion of the egg white diluted in water precipitated immediately, while the egg white diluted in buffer did not. Aliquots of each were centrifuged and samples were taken from the supernatants. Each sample yielded only one, but different, peak for lysozyme (Figure 3). The sample diluted in buffer produced a peak with the same migration time as the primary peak in the previous analyses. The sample diluted in water yielded the distinct companion peak.

From this, it was concluded that the companion peak is not an impurity in the standard protein, but possibly an unfolded form of lysozyme. This unfolding was reversed in the case of the standard sample mix, by raising the pH of the buffer. Whether this reversal is aided by the re-hydration of the lyophilized protein powder, and whether the same could be accomplished with water-diluted egg white, is as of yet undetermined.

To determine the effect of pH on the separation, the 100mM phosphate / 60 mM borate buffer was made at pH 7.0, 8.0, and 9.0, and analyses were performed on the CElect-P1 column (Figure 4). Under pH 7.0 conditions, the first (native) lysozyme peak was broader than at pH 8.0 and the conalbumin peak exhibited a tail, while the ovalbumin peak was slightly sharpend. At pH 9.0, no unfolded form of lysozyme was observed, and the response of the native lysozyme peak increased. However, the ovalbumin response was decreased. These results were as expected.

Using the pH 7.0 buffer creates a more acidic environment, which aids lysozyme degradation. In addition, pH 7.0 approaches the pI of conalbumin, making the protein less negative and more prone to adsorption to the capillary wall. This pH is still considerably above the pI

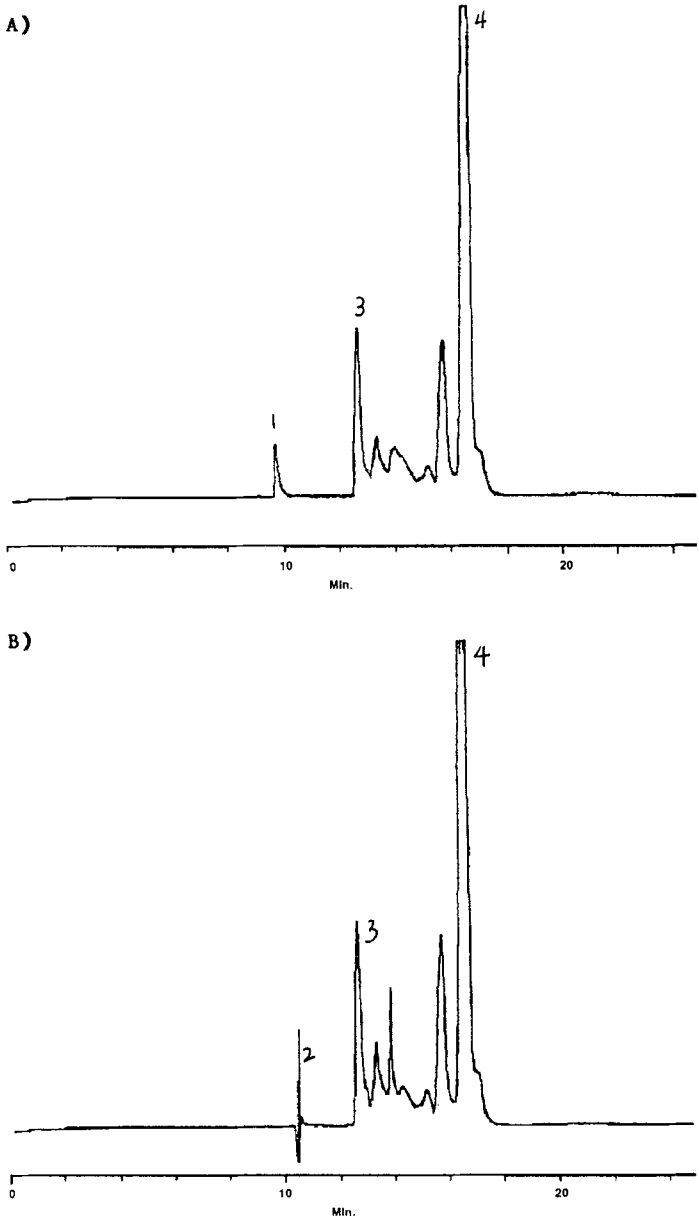

Figure 3: Egg white samples on a CElect-P1 column. Buffer: 100mM sodium phosphate / 60 mM sodium borate, pH 8.0. A) egg whites in separation buffer, B) egg whites in water. Peaks: 1) lysozyme (native) 2) lysozyme (unfolded) 3) conalbumin 4) ovalbumin.

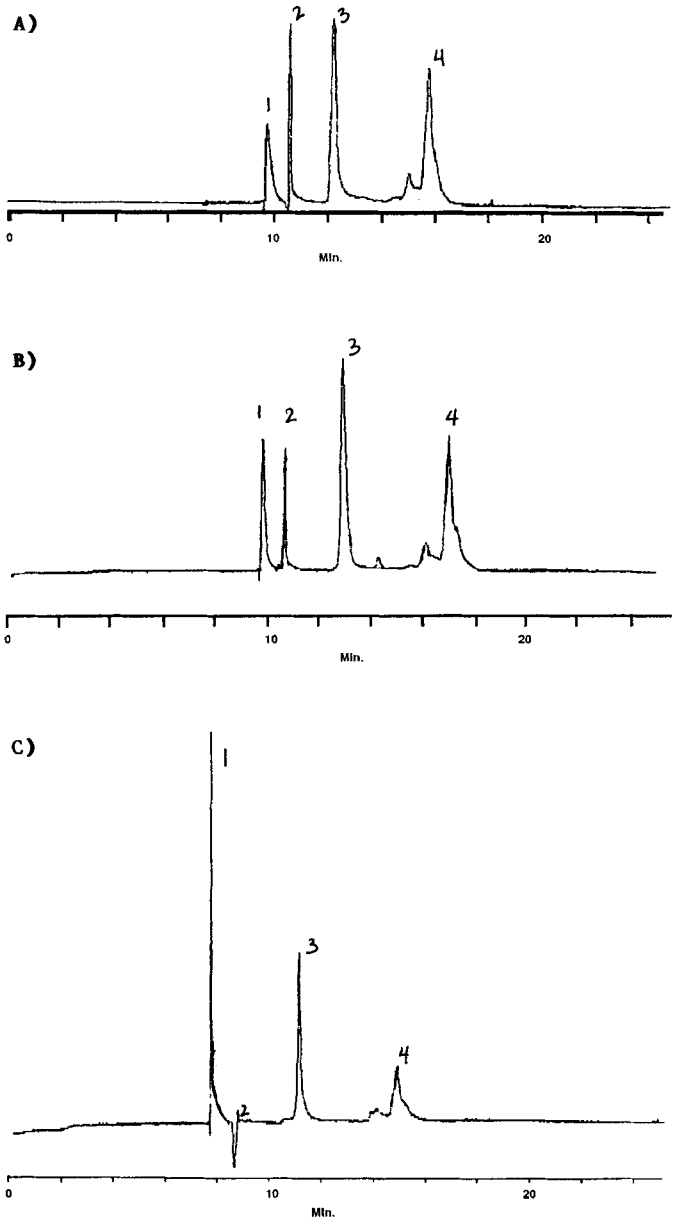

Figure 4: Optimizing pH for egg white proteins. Buffer: 100mM sodium phosphate / 60 mM sodium borate: A) pH 7.0, B) pH 8.0, C) pH 9.0. Peaks: 1,2) lysozyme 3) conalbumin 4) ovalbumin.

TABLE 2

Standard Deviations for Migration Time (Mt) and Peak Area for Protein Standards in Buffer, Using a CElect-P1 Column

Protein	%RSD (Mt)*	%RSD (Area)*
Lysozyme	0.5755	3.5798
Conalbumin	1.7157	3.9566
Ovalbumin	1.7741	3.0506

* n=9

of ovalbumin, and thus ovalbumin remains relatively unaffected. At pH 9.0, the more basic environment is better for lysozyme, and very little of the protein unfolds, resulting in one large peak of improved shape. Conalbumin, once again negative, returns to its previous condition, but ovalbumin is affected by the relatively high pH environment. The high pH causes ovalbumin to degrade (2), which is apparent in the decreased peak height. Repetitions of a sequence of analyses at varying pHs were made (in the order 7,8,9; 7,8,9; 7,8,9) using the same sample, to prove that ovalbumin degradation was caused by high pH rather than by thermal degradation due to standing at room temperature. All runs at each individual pH were reproducible.

Reproducibility of the peak area and migration time for the CElect-P1 column was studied over nine successive analyses, using the 100mM sodium phosphate / 60 mM sodium borate pH 8.0 buffer. The heat lability of this sample created some difficulty in the analysis. To help eliminate thermal degradation of the sample components, a new mix of standards was created in running buffer, and analyses were conducted with an ice-and-water-filled sample tray. The ice, sample, and buffer were replaced after every three separations. The percent RSD for migration time and peak area for each protein are shown in Table 2.

CONCLUSIONS

The most reproducible separation of the egg white proteins lysozyme, conalbumin, and ovalbumin, at near neutral pH, was achieved using a 100mM phosphate / 60 mM borate buffer, pH 8.0, on a CElect-P1 bonded phase column, as opposed to results on untreated fused silica and CElect-H bonded phase columns. The CElect-P1 column provided the high level of electroosmotic flow and hydrophilic coating necessary to perform the analysis in the desired time frame. A pH of 8.0 provided the best overall conditions, giving good peak shapes for lysozyme and maintaining the integrity of ovalbumin. Although these conditions produced results consistent with the objective, the method could be further optimized. For example, the effective length of the column could be shortened to reduce analysis time. This would also theoretically reduce the number of wall contacts over the time of the analysis, thus reducing the potential for adsorbtion.

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