High-Performance-Liquid Chromatography of *Thermus aquaticus* 50S and 30S Ribosomal Proteins

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Key Words

Column liquid chromatography
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Ribosome
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Gel electrophoresis

Summary

The ribosomal 50S and 30S subunit proteins (r-proteins) of *Thermus aquaticus* have, for the first time, been characterized by size exclusion chromatography (SEC) and by reversed phase high performance liquid chromatography (RPC). To ensure that the best resolution in the RPC was obtained, the elution conditions, such as gradient time, flow rate, temperature, ionic strength of the eluent and the type of stationary phase were optimized. Correlation between experimentally found retention times and those predicted by DryLab G was better than 0.7 % over 30 peaks. Protein fractions from RPC runs were desalted and processed by gel electrophoresis so that the ribosomal proteins could be identified by their position on SDS-polyacrylamide gels. The enhanced speed and quality of separation which has been achieved in this study is expected to bring advantages in experimental work with ribosomal proteins as well as with other biopolymers. In our case the high resolution technique provides a basis for the preparation of a collection of individual ribosomal protein components for future rRNA-protein interaction studies.

Introduction

Ribosomes play an important role in protein biosynthesis. The recent advances in the crystallization of ribosomal subunits make it feasible that in the future their structure will be determined by X-ray analysis [1]. In order to achieve this goal the primary structures of the ribosomal proteins must be established for which their high resolution separation is essential.

The 70S ribosomes of procaryontes consist of two dissociable subunits with sedimentation coefficients of 30S and 50S [2]. The 30S subunit is composed of a 16S rRNA and about 21 proteins, while the 50S subunit consists of a 23S rRNA, a 5S rRNA and approximately 34 proteins. The ribosomal 5S rRNA is at the 50S subunit and is known to interact specifically with three ribosomal proteins [2, 3]. The importance of the 5S rRNA in the functioning of the ribosome has been demonstrated by total reconstitution experiments, in which it was shown that ribosomal fragments which had lost their 5S rRNA had also lost their biological activity [4, 5].

Thermus aquaticus, a eubacterium, isolated from hot springs with temperatures between 70–75 °C, was first described by Brock and Freeze in 1969 [6]. In contrast to ribosomal proteins from *E.coli,* which are well characterized [7], *Thermus aquaticus* ribosomal proteins have, so far, not been separated by HPLC. Until now chromatograms of *Th. aquaticus* ribosomal proteins have exhibited broad peak shapes, and therefore ill-defined resolutions. In addition they have lacked any kind of reproducibility. The reason for this may be related to their thermostability, which is probably caused by strong intermolecular forces between the individual proteins.

The intention in this study was to improve the speed and resolution in ribosomal protein-HPLC, by separating the firmly bound proteins of the 50S and 30S subunits of *Thermus aquaticus* by preventing protein-protein-interactions during separation, and by introducing computer-supported techniques to ease the handling of individual peak components in the separation of complex protein mixtures.

Experimental

The isolation of 70S ribosomes and ribosomal subunits from *Thermus aquaticus,* typ YT1, was carried out according to the method of Cronenberg and Erdmann [8]. The subunit proteins were extracted at 0 °C with glacial acetic acid, containing 5 vol. % 1M magnesium acetate solution. The precipitated rRNA was pelleted by centrifugation (4 °C, 10000 RPM, 1hr). The supernatant was dialysed in

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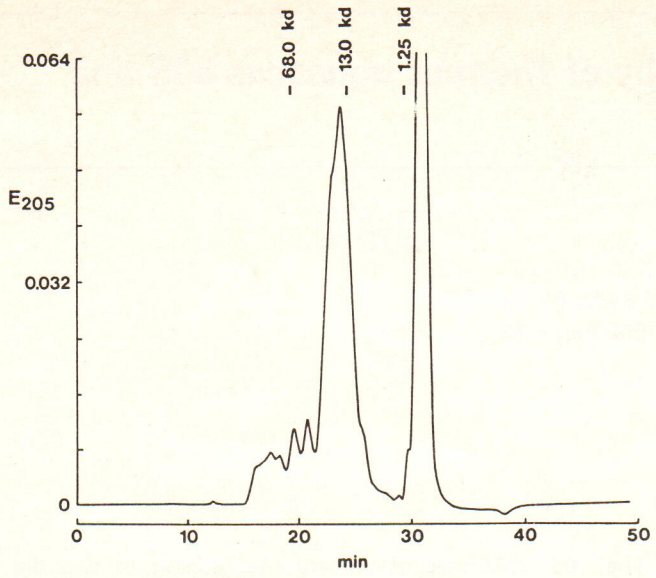

Figure 1

Separation of Thermus aquaticus 50S ribosomal proteins on Column 1 by SEC: 75 × 7.5 mm + 300 × 7.5 mm; eluent, 50 mM $(NH_4)_2SO_4$ 20 mM NaH_2PO_4, pH 5.0, flow rate: 0.5 ml/min; sample amount: 40 µg in 40 µl 2 % acetic acid.

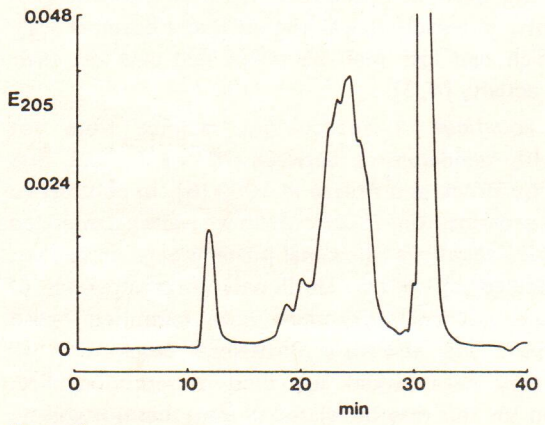

Figure 2

Separation of Thermus aquaticus 30S ribosomal proteins. Conditions as in Figure 1.

steps of 20 %, 10 %, 5 % to 2 % (w/w) acetic acid and kept at − 20 °C.

The molecular size distribution of subunit proteins 50S and 30S was measured by SEC (Figures 1, 2). The separation parameters of proteins under various conditions of RPC are given in the legends to the Figures.

Computer Simulation of Gradient Elution Runs

Retention times and peak areas of two gradient runs were evaluated and transferred to the DryLab G software, which is based on the work of Snyder et al. [9–11]. Prediction of retention times was checked experimentally on 30 peaks, 24 of these having molecular sizes between 9 and 24 kDa, measured by SDS-PAGE.

Determination of Purity of HPLC Peak Fractions

Fractions from HPLC runs were collected, desalted by HPLC [12], lyophilized and separated on SDS-gel (15 % acrylamide) for 10h.

Protein content was measured by a modified Lowry-technique as described by Smith et al. [13]. Silver staining was carried out by the method of Schwitzer et al. [14].

Equipment

We used a home-made HPLC apparatus consisting of two pumps, gradient-programmer, mixing chamber, (Knauer, Berlin); autosampler Promis, (Spark Holland, Emmen, Netherlands), high-temperature oven (Techlab, Braunschweig) spectrophotometer, dual-channel-recorder (Knauer, Berlin); detector/PC-interface (Nelson-Analytical, Zug, Switzerland); Personal Computer V286 (Victor, Frankfurt); printer NEC P6 (NEC, Berlin).

Software

Simulation of chromatograms was carried out using the software 'Drylab G' (LC-Resources, Lafayette, USA). For calculation of the retention time accuracy the software 'Open Access' (SPI, Munich) was used.

Measurement of retention times was carried out by the Nelson integration software. Peak areas for the simulation of chromatograms by DryLab were also taken from Nelson area tables.

Columns

The following columns were used:

Column No. 1.: SEC-column 75 × 7,5 mm and 300 × 7,5 mm (TSK250) (BioRad, Munich),

Column No. 2.: Hypersil Octyl 300 A 120 × 4,6 mm (Molnar, Berlin),

Column No. 3.: Ribosomal Protein Column 300 A 250 × 4,6 mm (Molnar, Berlin).

Chemicals

Aqueous buffer eluents were made by using NaH_2PO_4 and concentrated H_3PO_4 (Laborat, Berlin), HPLC-grade water and acetonitrile (Merck, Darmstadt) as organic modifier. All eluents were degassed by helium.

Results and Discussion

SEC-Separations

Proteins are separated rapidly on silica-based chemically modified stationary phases by size exclusion chromatography [15–18]. 50S ribosomal proteins of *Methanococcus vannelii* and *Escherichia coli* were separated by Kamp et al. on a TSK 2000 SW column, which resolved the protein mixture into 7 bands [19, 20].

Separation of the proteins of the 50S and 30S subunits of *Thermus aquaticus* has been completed by SEC on column 1. The separation is highly reproducible and the

size distribution depends on eluent pH and ionic strength. Eight bands of 50S proteins can be traced as shoulders with a range of molecular weight primarily around 20 kDa (Figure 1). The MW distribution of 30S proteins is similar to those of 50S, but somewhat broader (Figure 2).

SEC is a low resolution method and can only be used as a pre-fractionation technique. Its use only gives a rough estimation of the size distribution of the ribosomal proteins.

Separation of ribosomal proteins of thermophilic bacteria is difficult due to strong interactive forces between the proteins themselves. We therefore concentrated our efforts on high resolution HPLC-techniques.

Reversed phase separation of peptides [21, 22] and of proteins [23] on wide pore stationary phases with chemically-bonded hydrocarboneaceous ligands is a method with high resolution power and excellent reproducibility, which opens up new opportunities for conformational studies of proteins [24]. The fast kinetics of the mass transfer process are responsible for low band spreading and fast re-equilibration times in gradient elution.

Ribosomal proteins of *E. coli* on nonpolar stationary phases were first separated by Cooperman et al. [25] and by Kamp and Wittmann-Liebold [26]. These ribosomal proteins are fairly basic proteins, as shown by Kerlavage et al. [27] who reported an average isoelectric point of 10.11 ± 2.11 for 29 different 50S r-proteins.

Ghrist and Snyder recently investigated the design of optimized gradient runs for ribosomal proteins of *E. coli*, showing adequately reliable predictions of separation by gradient elution so that consuming trial and error experiments may be reduced [28]. The background and the theory of this optimization technique has been investigated in detail [29]. Recommendations for the best approach to designing an optimal gradient for a given sample are provided [30].

We started gradient elution experiments using Hypersil Wide Pore 300A, 5 µm, C-8-material in 120 × 4,6 mm columns (Figure 3).

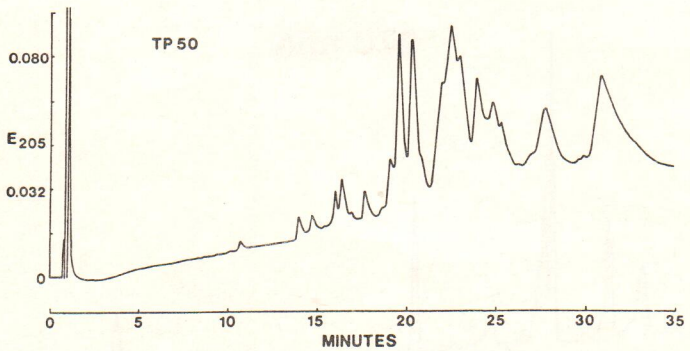

Figure 3

Separation of Thermus aquaticus 50S ribosomal proteins by reversed phase chromatography on Hypersil Wide Pore C8, 5 µm; 120 × 4.6 mm; eluent A: 50 mM H_3PO_4; eluent B: acetonitrile; gradient 5% B to 100% for 60 min; detector at 205 nm, detector sensitivity 0.16 AUFS., flowrate: 2.0 ml/min; sample size: 50 µg proteins in 50 µL of 2% acetic acid.

The results of the first two runs showed reproducible chromatograms. The peak tracking and assigning process however was adversely affected by broad peaks, changing peak sizes and changing retention order between the two gradient runs. The problem peaks were partially overlapping and tailing, similar to those of strong basic solutes in reversed phase chromatography (RPC) on insufficiently covered silica. These effects have been named "silanophilic interactions" by Horvath [31].

Changing Elution Conditions

First we tried to improve the resolution by reducing silanophilic interactions. Addition of 2 mM triethylamine (TEA) to the eluent changed little in comparison to the the previous runs. At least in principle the extent of peak overlapping remained the same. Further increase in TEA-concentration in the eluent was not considered to be worth while due to the difficulties in removing the compound from HPLC-fractions prior to subsequent investigations such as SDS-PAGE or amino acid sequencing.

Decreasing the gradient slope to 90 min and 180 min, which should, in general, improve separation, gave a similar low resolution, but a different peak pattern with extremely broad peaks. From these experiments it was obvious that the mass transfer process has slow kinetics, indicating the presence of ionic forces either between the protein species themselves or between protein and stationary phase.

Another possible reason for broad peak shape could have been bleeding of the support with a releasing of proteins from previous injections. Blank gradients however proved that this was not the case.

Temperature

Some biological macromolecules can exist in a large number of steric forms, which are in equilibrium with each other. The cyclic peptide cyclosporine shows this type of behaviour, giving broad peaks at room temperature. At 70 °C however, the peak from is becoming sharp as the speed of conformational changes increases. We therefore tried working at 80 °C but there was no improvement. In problem peak areas however peak pattern changes were clearly observed.

Urea

In an attempt to avoid reaggregation of individual subunit proteins, which could be held together by hydrophobic interactions, the TP 50 sample was incubated with 8 M urea. The subsequent gradient run, however, did not show any improvement in resolution.

Ionic Interactions

The obvious reason for the unacceptable peak formation was ionic interaction between the proteins, or between proteins and the stationary phase. Following our attempts to weaken solvophobic interactions using RPC in the gradient mode and applied elevated temperature, again with limited success, we finally assumed that there must be other forces, mainly ionic, between the protein species and

the stationary phase, which give rise to slow mass transfer kinetics. The fact that ribosomal proteins are easily soluble in concentrated acetic acid supported this hypothesis. We therefore concluded that high ionic strength mobile phases could weaken the unfavorable ionic interactions, leading to better peak shapes for the thermostable ribosomal proteins. This is a novel approach in RPC with organic solvents, since demixing and salt suspension formation quite often takes place, resulting in plugged columns. Consequently we tried to find mixtures of water and acetonitrile of high ionic strength and with good miscibility with the organic modifier. We found, experimentally, that a mixture containing equal volumes of acetonitrile and 0.5 M phosphate buffer (pH 2.1) as eluent B is perfectly miscible with a 1 M phosphate buffer (pH 2.1) as the aqueous A buffer.

Subsequently the ionic strength of the aqueous mobile phase was varied from the original low and unsuccessful value of 50 mM phosphate (pH 2.1) in four steps to 250, 500, 750 and 1000 mM. The final result is shown in Figure 4, in which a distinct improvement in resulution is demonstrated. Although, in Hydrophobic Interaction Chromatography (HIC), retention normally increases with the ionic strength of the eluent, here the opposite was the case: retention time is reduced. At the same time the chromatogram became more structured with increased peak sharpness.

At this point it is unclear whether the reason for the low speed of the mass transfer kinetics is the ionic interaction between the ribosomal proteins, or between the proteins and the stationary phase.

With a column of a different type (No. 3), further improvement in resolution could be achieved in the separation of ribosomal proteins TP50 and TP30 as shown in Figure 5 and Figure 6. This resolution was sufficient for collection of fractions for further studies. For this purpose we chose two different gradient run times: 90 and 270 min, at which peak tracking was practicable.

In gradient elution of complex protein mixtures peak position is rather difficult to predict because band movement is a function of molecular size. Resolution between different sized species is also time consuming to calculate [10, 11].

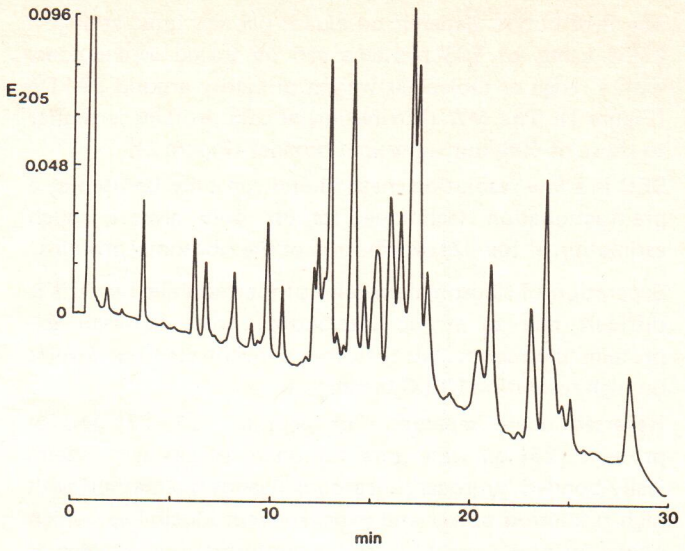

Figure 4

Separation of Thermus aquaticus 50S ribosomal proteins by reversed phase chromatography on column 2 using 1000 mM phosphate buffer (pH 2.1) as eluent A. As eluent B a mixture of acetonitrile: water (50:50) (V:V) was used. Flow rate: 2,0 mL/min, pressure: 9,9 MPa. Gradient elution from 36% B to 100% B in 30 min. Detector: 0.16 AUFS at 205 nm. Protein amount: 50 µg.

To predict the retention times of ribosomal proteins we used DryLab G. For this purpose two actual basic experiments had to be carried out, namely two gradient runs, differing in gradient time by a factor of 3. After entering two sets of retention times of a 90 min and of a 270 min gradient run with 30 bands in each run and a set of experimental parameters into DryLab G, we found the standard deviation of the difference between predicted and experimental retention times to be less than 0.7 % (Table I).

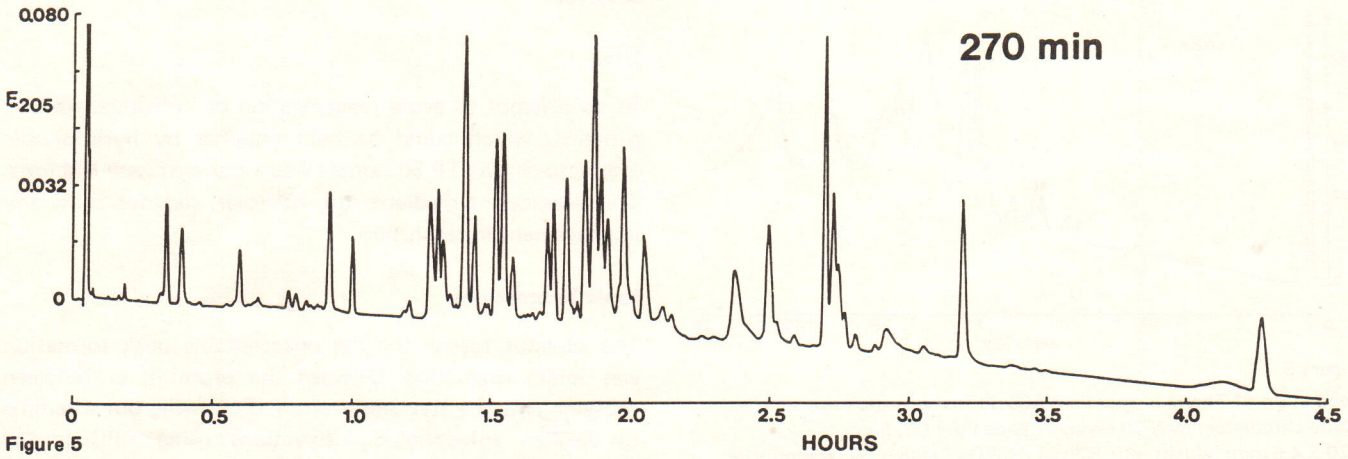

Figure 5

Separation of Thermus aquaticus 50S ribosomal proteins by reversed phase chromatography on column 3: 250 X 4.6 mm, 10 µm. Eluent A: 1 M phosphate buffer (pH 2,1); flow rate: 1.5 mL/min, pressure: 18 MPa; Eluent B: (CH_3CN): (500 mM phosphate buffer, pH 2.1) (50:50) (V:V); gradient: from 50 % B linear to 100 % B for 270 min; flow-rate, 1.5 ml/min; 100 µg were injected in 100 µl of 2 % acetic acid.

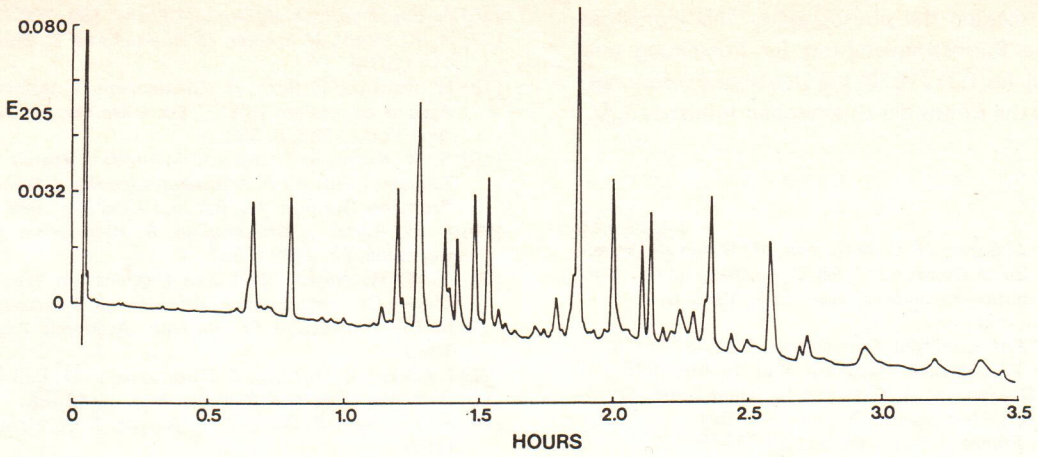

Figure 6

Separation of Thermus aquaticus 30S ribosomal proteins by reversed phase chromatography. Conditions as in Figure 4.

Table I. Comparison of predicted and experimentally found retention times for TP 50 of thermus aquaticus ribosomal proteins.

R1 90 min	R2 270 min	R3 Pred. 180 min	R3 Exper. 180 min	Diff. R3 min	D. abs min	Diff. in %
11.53	20.05	16.36	15.97	0.28	0.28	1.71
13.38	23.32	19.07	18.65	0.32	0.32	1.68
17.40	35.80	27.35	26.75	0.60	0.60	2.19
20.32	46.40	33.98	33.47	0.51	0.51	1.50
22.78	55.22	39.51	38.88	0.63	0.63	1.59
24.57	60.15	42.90	42.32	0.58	0.58	1.35
29.18	76.97	53.38	52.73	0.65	0.65	1.22
29.75	78.68	54.52	53.88	0.64	0.64	1.17
30.27	79.83	55.39	55.32	0.07	0.07	0.13
31.42	84.00	57.99	57.50	0.49	0.49	0.84
34.28	86.60	61.19	60.72	0.47	0.47	0.77
34.45	91.30	63.30	62.55	0.75	0.75	1.18
34.60	92.90	64.08	63.25	0.83	0.83	1.30
35.08	93.50	64.69	63.62	1.07	1.07	1.65
36.02	94.91	65.97	65.40	0.57	0.57	0.86
38.37	102.28	70.81	70.77	0.04	0.04	0.06
38.95	103.55	71.77	70.77	1.00	1.00	1.39
39.45	106.35	73.32	72.82	0.50	0.50	0.68
40.67	110.35	75.90	75.42	0.48	0.48	0.63
41.15	112.30	77.08	76.50	0.58	0.58	0.75
41.78	113.85	78.19	77.40	0.79	0.79	1.01
42.33	115.23	79.18	78.78	0.40	0.40	0.51
43.23	118.57	81.25	80.70	0.55	0.55	0.68
51.15	142.67	97.23	97.43	−0.20	0.20	0.21
57.98	161.97	110.39	110.32	0.07	0.07	0.06
58.63	163.80	111.64	111.63	0.01	0.01	0.01
59.00	164.00	112.00	112.25	−0.25	0.25	0.22
59.67	166.20	113.42	113.53	−0.11	0.11	0.10
67.98	191.82	130.39	130.25	0.14	0.14	0.11
92.35	256.40	175.48	175.25	0.23	0.23	0.13
				Mean	0.46	0.86
				St. dev.	0.28	0.62

Molecular weight data of some of the 50S ribosomal protein bands has been investigated by SDS-PAGE. The observed range of molecular weights was between 9 and 23 kDa for 24 isolated 50S proteins.

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