

RETICULOCYTE GROWTH FACTOR AND PREPARING METHOD AND APPLICATION THEREOF

BACKGROUND

Technical Field

5 The present invention relates to the field of medical biotechnology, and in particular relates to a reticulocyte growth factor and a preparing method and application thereof.

Related Art

At present, the most efficient medicine for clinically treating severe anemia is Erythropoietin (rhu-EPO). Since the recombinant human (rhu) EPO of the Amgen
10 Company in America was approved by FDA in 1989, the rhu-EPO has been generally commonly accepted, and has developed to the most successful example in DNA recombinant medicine. EPO is one unique special red cell increasing factor in generation of red cells as so far. Although it is reported that Stem Cell Growth Factor (SCF) and Colony Stimulating Factors (CSFs) also have a promoting action for the red cells, their functions
15 are diversified and not exclusive. At present, no new medicine for treating the severe anemia is found yet.

SUMMARY

The present invention aims to provide a new cell growth factor - Reticulocyte Growth Factor (RGF), and a preparing method and application thereof.

20 In a research process of the present invention, in kidney cell culture fluid, proteins with erythropoiesis activity exist in both human urine and plasma, but their molecular weights, immunogenicity and even receptors are different from EPO. The content of such active proteins is very low, it is difficult to obtain enough pure products for quality research, and finally, it is found that impurities from an α_1 -antitrypsin preparation of the human
25 plasma has high activity for promoting generation of reticulocyte. Several high-activity proteins, obtained by biochemical and purifying means such as High Performance Liquid Chromatography (HPLC), are peptide fragments of different lengths in a precursor of the

α_1 -antitrypsin, and have carbohydrates of different lengths, and some components have few or no carbohydrate.

Technical solutions of the present invention

1. Purification of RGF

5 a, the α_1 -antitrypsin preparation (A-9024) of Sigma Company is from human plasma, and has very high activity for promoting generation of the reticulocyte. Activity inspection adopts a mouse reticulocyte determining method of professor CHENG Yaqin, which is described as follows:

animals: ICR mice, weight: 18-22kg, male

10 samples: diluting according to the activity of RGF, 5-20 μ g/ml in common

method: dividing three mice in one group, intramuscularly injecting 0.06ml, 0.12ml and 0.24ml, injecting for three days continuously, sampling eye socket blood on the fourth day, adding 7-8 drops into a tube with a lid filled with 20 μ l 100U/ml of heparin sodium, mixing uniformly, taking out 50 μ l blood, dropwise adding on dried brilliant cresyl blue dye
15 precoated at one end of a glass slide, fully mixing, and placing in a 37°C wet box for 30 minutes; pushing single layer of cells on a second glass slide, after naturally drying, fixing with methyl alcohol, then redyeing through Wright's stain, dropwise adding 0.02mol/LPBS (PH6.8), avoiding drying up, and washing the dye liquor with PBS or distilled water after 30 minutes;

20 reading a film by a high power oil immersion lens, where a nucleus substance in the reticulocyte can be dyed to be deep blue color dot-like, line-like, chain-like or cluster-like, while the red cells do not contain such substance; counting 500-1000 red cells in common and calculating the percentage of the reticulocyte; reading two films for each sample, averaging, then averaging again for three mice in each group, and subtracting the
25 percentage of the reticulocyte in a control group (0.12 μ l normal saline) to obtain the net increase percentage of the reticulocyte, defining every 1% of increase as one unit (U), and calculating specific activity by injected protein content (once), namely taking the rising unit stimulated by each mg of protein (U/mg) as the specific activity;

preparing the α_1 -antitrypsin preparation into different concentrations with normal saline, taking 15 ICR male mice (weight: 18-22g), dividing into 5 groups each of which contains 3 mice, intramuscularly injecting 0.12ml normal saline into the control group, injecting 0.12ml antitrypsin preparation of different concentrations into the rest group, 5 mice, injecting for three days continuously, sampling eye socket blood on the fourth day, dyeing by brilliant cresyl blue, and calculating the percentage of the reticulocyte in the red cells with the oil immersion lens, wherein a result shows that the activity for promoting the generation of the reticulocyte in the antitrypsin preparation is very high, and can serve as an initial material for further separation and purifying. The result is as shown in Table 1:

10 Table 1 Activity for promoting generation of reticulocyte in α_1 -antitrypsin preparation

α_1 -antitrypsin preparation concentration	Reticulocyte%	Net increase reticulocyte%
0	1.8	
2 μ g/ml	8.2	6.4
5 μ g/ml	6.6	4.6
20 μ g/ml	16.45	14.65
80 μ g/ml	1.8	0

The data indicates that the α_1 -antitrypsin preparation indeed contains higher activity for promoting generation of reticulocyte.

b, dissolving the α_1 -antitrypsin into 2mg/ml with PBS of 0.02mol/L, performing neutral separating by an HPLC C₁₈ reversed-phase column to obtain 3 peak groups, wherein, 15 through determining, only the 3rd peak group has activity, a C18 reversed-phase column separation pattern is as shown in FIG. 1 and a result is as shown in Table 2:

Table 2 Activities of respective parts obtained by separating the α_1 -antitrypsin through the HPLC C₁₈ reversed-phase column

Collected parts	Reticulocyte %	Net increase reticulocyte%
Control saline 0.12ml	2.4	
Peak 1 20 μ g/ml 0.12ml	3.3	0.9
Peak 2 20 μ g/ml 0.12ml	4.48	2.08

Peak 3 20µg/ml 0.12ml	8.6	6.2
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c, increasing an eluting gradient of the peak 3 group with the C18 reversed-phase column again to obtain 10 collected parts, wherein an activity inspection result shows that the 8th collected part has the highest activity, the 7th and 9th collected parts have the activity second to that of the 8th collected part, and the other 7 collected parts have no activity. A separating pattern is as shown in FIG. 1, and an activity determining results are shown in Table 3:

Table 3 Activities of 7[#], 8[#], 9[#] and 10[#] parts in the 10 collected parts obtained by separating the third peak for the second time

Collected parts	Reticulocyte %	Net increase reticulocyte%
Control saline 0.12ml	1.2	
Peak 7 [#] 20µg/ml 0.12ml	4.9	3.7
Peak 8 [#] 20µg/ml 0.12ml	8.3	7.1
Peak 9 [#] 20µg/ml 0.12ml	5.8	4.6
Peak 10 [#] 20µg/ml 0.12ml	4.1	2.9

d, performing preparative SDS-PAGE (as shown in FIG. 3) on the eighth collected part, cutting a piece of gel, according to the position displayed by staining, for the undyed gel, cut first to fourth bands of gel, respectively triturating, extracting with water for 24 hours, extracting for three times continuously, combining supernate, determining activity after freeze-drying, wherein, only the bands 1, 2 and 3 extracting liquor show activity, the band 4 has no activity, a result is as follows:

Table 4 Activities of extracting liquors of respective bands cut by electrophoresis and prepared by the activity peak eighth sample after HPLC separation

Prepared electrophoresis bands	Reticulocyte %	Net increase reticulocyte%
Control saline 0.12ml	1.2	
Band 1 20µg/ml 0.12ml	10.7	9.2
Band 2 20µg/ml 0.12ml	8.1	6.6
Band 3 10µg/ml 0.12ml	7.2	5.7

Band 4 20μg/ml 0.12ml	1.7	0.2
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then performing SDS-PAGE inspection after freeze-drying of the plurality of extracting liquors, wherein the bands 1 and 3 have clear dyed strips, the band 2 is high in activity but is close to the band 1, possibly has pollution, is fuzzily dyed, and is obvious not single bands, therefore, samples of the bands 1 and 3 are sent to the Suzhou ProtTech Inc. for sequencing, an identification result shows that the bands 1 and 3 are a precursor of the α_1 -antitrypsin, we called the peptide fragment in such family as Reticulocyte Growth Factors (RGF family), and the sequence of the precursor is:

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Met Pro Ser Ser Val Ser Trp Gly Ile Leu Leu Leu Ala Gly Leu
 1              5              10              15
Cys Cys Leu Val Pro Val Ser Leu Ala Glu Asp Pro Gln Gly Asp
              20              25              30
Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Gln Asp His Pro
              35              40              45
Thr Phe Asn Lys Ile Thr Pro Asn Leu Ala Glu Phe Ala Phe Ser
              50              55              60
Leu Tyr Arg Gln Leu Ala His Gln Ser Asn Ser Thr Asn Ile Phe
              65              70              75
Phe Ser Pro Val Ser Ile Ala Thr Ala Phe Ala Met Leu Ser Leu
              80              85              90
Gly Thr Lys Ala Asp Thr His Asp Glu Ile Leu Glu Gly Leu Asn
              95              100             105
Phe Asn Leu Thr Glu Ile Pro Glu Ala Gln Ile His Glu Gly Phe
              110             115             120
Gln Glu Leu Leu Arg Thr Leu Asn Gln Pro Asp Ser Gln Leu Gln

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	125		130		135
Leu Thr Thr Gly Asn Gly Leu Phe Leu Ser Glu Gly Leu Lys Leu					
	135		140		145
Val Asp Lys Phe Leu Glu Asp Val Lys Lys Leu Tyr His Ser Glu					
	150		155		160
Ala Phe Thr Val Asn Phe Gly Asp Thr Glu Glu Ala Lys Lys Gln					
	165		170		175
Ile Asn Asp Tyr Val Glu Lys Gly Thr Gln Gly Lys Ile Val Asp					
	180		185		190
Leu Val Lys Glu Leu Asp Arg Asp Thr Val Phe Ala Leu Val Asn					
	195		200		205
Tyr Ile Phe Phe Lys Gly Lys Trp Glu Arg Pro Phe Glu Val Lys					
	210		215		220
Asp Thr Glu Glu Glu Asp Phe His Val Asp Gln Val Thr Thr Val					
	225		230		235
Lys Val Pro Met Met Lys Arg Leu Gly Met Phe Asn Ile Gln His					
	240		245		250
Cys Lys Lys Leu Ser Ser Trp Val Leu Leu Met Lys Tyr Leu Gly					
	255		260		265
Asn Ala Thr Ala Ile Phe Phe Leu Pro Asp Glu Gly Lys Leu Gln					
	270		275		280
His Leu Glu Asn Glu Leu Thr His Asp Ile Ile Thr Lys Phe Leu					
	285		290		295
Glu Asn Glu Asp Arg Arg Ser Ala Ser Leu His Leu Pro Lys Leu					
	300		305		310
Ser Ile Thr Gly Thr Tyr Asp Leu Lys Ser Val Leu Gly Gln Leu					
	315		320		325
Gly Ile Thr Lys Val Phe Ser Asn Gly Ala Asp Leu Ser Gly Val					
	330		335		340
Thr Glu Glu Ala Pro Leu Lys Leu Ser Lys Ala Val His Lys Ala					

				345					350					355
Val	Leu	Thr	Ile	Asp	Glu	Lys	Gly	Thr	Glu	Ala	Ala	Gly	Ala	Met
				360					365					370
Phe	Leu	Glu	Ala	Ile	Pro	Met	Ser	Ile	Pro	Pro	Glu	Val	Lys	Phe
				375					380					385
Asn	Lys	Pro	Phe	Val	Phe	Leu	Met	Ile	Glu	Gln	Asp	Thr	Lys	Ser
				390					395					400
Pro	Leu	Phe	Met	Gly	Lys	Val	Val	Asn	Pro	Thr	Gln	Lys		
				410					415					

2. Chemical properties of RGF

In order to determining the chemical properties of the bands 1 and 3 prepared by SDS-PAGE, the gel bands displayed by staining were sent to the Suzhou ProtTech Inc. for determining the sequences of the N end and C end, a result shows that the band 1 (RTF-6 in a sequencing report of the Suzhou ProtTech Inc.) contains four components which are fragments of different lengths of the precursor of the α_1 -antitrypsin, N end sites have slight difference, the C ends are same as the C ends of precursor, band 3 (RTF-8 in a report of the Suzhou ProtTech Inc.) contains only one component, the N end and C end are totally same as the bands 1-2 and contain 394 amino acids in total from the 25 site to the 418 site, the band 1 has a molecular weight of about 50000, which is 10000 larger than the band 3, it is known that the band 1 contains carbohydrate, the components in the band 1 are glycoproteins, the band 3 does not contain carbohydrate through speculation from the molecular weight, or contains extremely less carbohydrate.

The RGF family is a group of protein fragments with different lengths and different carbohydrate contents from the precursor of the α_1 -antitrypsin. An amino acid sequence thereof is:

N end (band1-1)

24

Ala Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr
 Ser His His Asp Gln Asp His Pro Thr Phe Asn Lys

49

N end (band 1-2)

25

Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser

N end (1-3)

27

Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His His
 Asp Gln Asp His Pro Thr Phe Asn Lys

5

49

N end (band 1-4)

30

Asp Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Gln Asp
 His Pro

Thr Phe Asn Lys

49

C fragment

412

Val Val Asn Pro Thr Gln Lys

418

10

3. Biological properties of RGF

a, therapeutic action of the RGF for aplastic anemia mice, this experiment is entrusted to the Pharmacology Teaching and Research Department of the Institute of Traditional Chinese medicine, Jiangsu,

modeling: Injecting 100mg/kg of cyclophosphamide ip into male mice of 18-22g for three continuous days

test: the mice are divided into 5 groups and EPO is domestic (NING Hongxin). The RGF is the produc prepaed in this laboratory, and a result of RBC and WBC examining
5 after continuous injecting for 8 days is as shown in Table 5:

Table 5 Therapeutic action of the RGF to aplastic anemia mice

Groups	Mouse sample number	Dosage	Result		Red cell promotion %	Leukocyte promotion %
			RBC	WBC		
Blank control	20	Normal saline	487±	190±		
			149.3	71.8		
Aplastic anemiamodel group	20	Normal saline	305±102.5	115±36.5		
EPO group in model group	20	333U/kg	429±94.8	201±79.6	40.66	77.78
RGF group in model group	20	167μg/kg	442±105.1	195±81.1	40.92	69.57
EPO group +RGF group in model group	20	1/2EPO±1/2RPF	516±108.4	233±104.5	69.18	102.61

The cyclophosphamide can obviously cause anemia to the mice, gneration of the red cells can be promoted by injecting the EPO or RGF, for example, the therapeutic action, achieved by half amount of EPO and half amount of RGF, for co-treating the generation of
10 the red cells is better than the therapeutic action achieved by single whole amount of the RGF or EPO, an obvious synergistic effect is achieved, and it should be noted that the RGF or EPO further has a promotion action for the generation of leukocytes, and have the same synergistic effect.

b, synergistic effect of the RGF and EPO for generation of mouse reticulocyte
15 animals: 30 ICR mice, male, weight: 18-22g, 10 groups, 3 mice in each group

experiment: preparing the RGF and EPO into three following concentrations: (a) RGF5 μ g/ml; (b) EPO (rhuEPO of Amgen Company) 18U/ml; (c) balanced mixing of the RGF and EPO; injecting the RGF and EPO with the concentrations of the a, b and c into the mice in each group by 0.06ml, 0.12ml and 0.24ml for continuous 3 days, sampling eye socket blood on the fourth day, smearing, dyeing, and examining the increase percentage of the reticulocyte with a microscope, wherein a result is as shown in Table 6:

Table 6 Synergistic effect of RGF and EPO (Amgon Company)

Injecting dosage (each mouse every day)	Net increase reticulocyte%		
	EPO (18U/ml)	RGF (5 μ g/ml)	EPO9U/ml RGF2.5 μ g/ml
0.06ml	4.8%	4.9%	5.3%
0.12ml	6.2%	6.6%	7.2%
0.24ml	7.4%	7.8%	11.3%

The RGF and EPO both have well activity for promoting the generation of the reticulocyte, 18U/ml of EPO and 5 μ g/ml of RGF are very close in activity, after the EPO and the RGF are mixed in the same amount, an obvious synergistic effect is generated, especially, the test group injected with the 0.24ml is higher than the test group injected with the 0.24ml of the EPO or RGF by about 50% in reticulocyte increase, then is this phenomenon possibly the false appearance caused by the generation of the mouse EPO caused by the RGF in vivo, for this purpose, we design a process that after the RGF of different dosages is injected to the mice for 3 days, the EPO level in blood of the mice is determined by monoclonal antibody to compare with the normal saline control group, the EPO level is not increased, so that such possibility is excluded. It is inferred that the EPO and the RGF act in different development stages of the red cells, under a relay action of the EPO and the RGF, the speed of the red cells is naturally increased. This hypothesis is verified in follow fluorescence labeling experiment.

The synergistic effect of the RGF and EPO occurs in the two experiments, and this phenomenon has great significance in treatment of the severe anemia and indicates a potential of more reasonably and more effectively treating the severe anemia.

c, fluorescence labeling experiment

labeling the EPO with fluorochrome rhodamine (RB200), wherein the EPO is red under the observation of a fluorescence microscope, labeling the RGF with fluorochrome FITC, wherein the RGF is green under the observation of the fluorescence microscope, sampling about 2ml of bone marrow cells from long bones of four mice, centrifuging at 1000r/m for four minutes, pouring off supernatant, resuspending in 6ml of Hank'S liquid, separating through a lymphocyte separating medium, and taking and centrifuging interface cells; diluting the cells with RPMI1640 into 1.8×10^6 /ml of cell suspension, taking 5ml of cell suspension, centrifuging, resuspending in 0.3ml of PBS, adding 60 μ l of FITC-RGF and 60 μ l of RB200-EPO, placing in a refrigerator for 30 minutes, wherein, by observing under the fluorescence microscope, most cells are doubly labeled, a few cells are singly labeled by FITC-RGF, but there are no cells labeled by RB200-EPO. This phenomenon prompts that the RGF is used in an earlier stage of a cell development process, an EPO receptor does not occur till the cells are developed to the next stage under the promotion of the RGF, therefore, the dual-receptor cells on which the RGF and EPO show at the cells occur.

Beneficial effects of the present invention

Differentiation and maturation of the red cells are a multi-stage multifactor-participated complicated process, however, a second factor for promoting the generation of the red cells is not found for last 20 years, so that the finding of the reticulocyte growth factor has great significance. When the reticulocyte growth factor and EPO are combined in use, the effect is better than that when the reticulocyte growth factor or EPO is singly used. It is indicated that different development stages of the red cells need different induction and promotion of different factors, which is a new start for us to learn the total development process of the red cells, and treat the severe anemia. The present invention helps to explain the synergistic effect of the RGF and EPO occurring in normal mice and aplastic anemia mice. Because they act in different development stages of the red cells, and bone marrow cells generate more EPO receptors under the action of the RGF, and thus are developed to dual-receptor cells, the EPO then continuously acts, and the better effect can be understood under a relay action. The invention develops a new medicine for

treating the severe anemia.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a chromatography pattern of an α_1 -antitrypsin separated by an HPLC C₁₈ reversed-phase column in first separation, wherein 3 collected parts are obtained from the first separation, and the 3rd part has activity.

FIG. 2 is 10 collected parts obtained by the HPLC C₁₈ reversed-phase column in second separation larger than the first separation by gradient, wherein the eighth peak collected part pointed by the arrow has higher activity.

FIG. 3 is an SDS-PAGE diagram of an eighth part in the ten collected parts in the second HPLC separation, wherein a molecular weight is about 50000, the part dyed with the deepest color is band 1 (RGF-B1), the molecular weights of about 40000 are called as band 3 (RGF-B3), the part between the B1 and B3 is called as RGF-B2, and the parts above B1 have no activity.

DETAILED DESCRIPTION

1, taking two bottles of α_1 -antitrypsin, dissolving in pH7.0 0.02MOL/L of PBS with the concentration of 2.5mg/ml, performing neutral separation by an High Performance Liquid Chromatography (HPLC) C18 column, and obtaining 3 peak groups, wherein, as shown in FIG. 1, the 3rd peak group shows a strong action for promoting the generation of reticulocyte;

2, re-separating the third peak group, but pulling the separated eluting gradient, and obtaining 10 collected parts, wherein only the 7th, 8th and 9th collected parts show the activity for promoting the generation of the reticulocyte, the 8th collected part has the highest activity as shown in FIG. 2 and FIG. 3; performing preparative SDS-PAGE on the 8th collected part, longitudinally cutting a piece of gel, dyeing by Coomassie Brilliant Blue to determine the position of proteins, wherein a deeper and wider band occurs at the position of the molecular weight of about 50000; cutting the corresponding gel block as band 1, cutting a clear band at the position of the molecular weight of about 40000 as band 3, wherein, 3-4 dyed bands between the band 1 and band 3 are fuzzy, one of the bands is

close to number 1, and is called as band 2, and the band between the molecular weights of 50000-68000 is called as band 4; crushing the gel of the four parts with glass rods individually, extracting for 24 hours with water, sucking out supernatant, and repeating for three times; combining and freeze-drying the extracting liquors obtained at four times on
 5 the 5th day, and dissolving with a small amount of water and then determining the activity, wherein a result shows that the extracting liquors of the bands 1, 2 and 3 have high activity, and the band 4 has no activity, and the result is as shown in table 3;

4, performing SDS-PAGE on the extracting liquors of the bands 1, 2 and 3, wherein the bands 1 and 3 have clear strips after staining, the band 2 has fuzzy strips, some strips are
 10 very close to the band 1 in position, therefore, the dyeing gel of the bands 1 and 3 are sent to the ProtTech Inc. for sequencing, a determining result shows that the bands 1 and 3 are parts of a precursor of the α_1 -antitrypsin and a result is as shown in the amino acid sequence;

5, in order to determine the chemical properties of the bands 1 and 3, the SDS-PAGE,
 15 drying gel blocks of the band 1 and band 3 are sent to the Suzhou ProtTech Inc. for sequencing, and a result is as shown in attachment 2. The N end and C end sequencing result shows that four components contained in the band 1 are fragments of different lengths in the precursor of the α_1 -Antitrypsin, the precursor is composed of 418 amino acids, these fragments have different start points of the N ends, and are respectively 24, 25,
 20 27 and 30 site amino acids; the C ends are same as the tail ends of the precursor. The band 3 contains single component, is totally same as the band 1-2 in structure and is composed of 394 amino acids in total from the 25th site to the 418th site. But the molecular weight is smaller than band 1 that contains carbohydrate, and based on the molecular weight, the band 3 contains no or extremely less carbohydrate;

25 6, the sequence of the precursor of the α_1 -Antitrypsin is known, the molecular weight of antitrypsin is known as 55000 – 56000, the bands 1 and 3 are both smaller than the precursor in molecular weight, and plural components between the molecular weights of 50000-40000 also show activity. It is estimated that the precursor of the Antitrypsin can be degraded into a group of peptide fragments in different lengths, and these peptide fragments

also have carbohydrate chains of different lengths or no carbohydrate. It is still unknown yet that whether the active protein groups composed of the peptide fragments have different action sites, but all the components of the antitrypsin family participate in the action of regulating the generation of the red cells in organism together with the EPO.

CLAIMS

What is claimed is:

1. A reticulocyte growth factor, characterized in that an amino acid sequence is:
 fragments in 418 amino acids of a precursor of α_1 -antitrypsin, which belong to the same
 5 family

N end (band 1-1)

24

Ala Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His
 His Asp Gln Asp His Pro Thr Phe Asn Lys

49

or

N end (band 1-2)

25

Glu Asp Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His His
 Asp Gln Asp His Pro Thr Phe Asn Lys

49

10

or

N end (band 1-3)

27

Pro Gln Gly Asp Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Gln
 Asp His Pro Thr Phe Asn Lys

49 或

or

15

N end (band 1-4)

30

Asp Ala Ala Gln Lys Thr Asp Thr Ser His His Asp Gln Asp His Pro
 Thr Phe Asn Lys

49

or

C end

412

Val Val Asn Pro Thr Gln Lys

418

all glycoprotein and non-glycoprotein with the 70% of sequence same as above sequence belong to the RGF family.

2. A preparing method of the reticulocyte growth factor according to claim 1,
5 characterized in that by comprising following steps:

(1) dissolving α_1 -antitrypsin preparation from human plasma into 2.5mg/ml with PBS of 0.02mol/L;

(2) separating through an HPLC C_{18F} reverse-phase column to obtain 3 protein peak groups, wherein through activity determining, the third peak group has activity of
10 promoting the growth of reticulocyte

(3) separating the third peak group through the HPLC C_{18F} again, wherein 10 protein collected parts are obtained when an eluting gradient is increased, and the 8th part has higher activity through activity determining;

(4) performing preparative SDS-PAGE on the 8th collected part, cutting gel with the
15 obvious protein and crushing, repeatedly extracting with water, detecting purity and activity after concentration, and sequencing.

3. Application of the reticulocyte growth factor according to claim 1 to the preparing of medicine treating anemia.

4. Application of the reticulocyte growth factor according to claim 3 to the preparing
20 of medicine treating anemia, characterized in that the reticulocyte growth factor and erythropoietin are combined for use, and have an obvious synergistic effect.